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Regulation of mouse blastocyst adhesion, outgrowth and secretion of matrix metalloproteinase-2 by cGMP and nitric oxide *in vitro*

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Abstract **Nitric oxide (NO) is a multifunctional messenger molecule produced through oxidation of L-arginine to L-citrulline by enzyme NO synthase (NOS). In the current study, mouse blastocysts were cultured in the different media, and the implantation capacity of blastocyst was evaluated by evaluating the percentage of embryos adhesion and outgrowth after culture for 12, 24 or 48 h. Matrix metalloproteinase-2 (MMP-2) mRNA was detected by RT-PCR, and MMP-2 protein was detected by gelatin zymography. Inhibition of blastocyst adhesion and outgrowth was observed in embryo cultured with 500 μmol/L NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) alone; however, 100 μmol/L S-nitroso-N–acetylpenicillamine (SNAP), a NO donor, and 20 μmol/L cGMP analogue, 8-Br-cGMP could block this inhibition. The expression and production of MMP-2 in the blastocysts were suppressed by L-NMMA, and SNAP or 8-br-cGMP could reverse this suppression. These results suggest that NO induces embryo implantation by cGMP signaling pathway.**

Keywords: nitric oxide, cyclic guanosine monophoshate, adhesion, outgrowth, implantation, matrix metalloproteinase-2.

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The embryo implantation requires extensive remodeling of the maternal extracellular matrix (ECM) so that the embryonic cells can invade the uterus wall to make contact and merge with the maternal vascular network. Implantation is the physical adhesion of the embryo to the uterus, and although it involves relatively few cell types, the reciprocal signaling relationships between maternal and fetal tissues can be very complex[1].

Nitric oxide (NO), which is a colorless gas synthesized from L-arginine by NO synthases and readily disperses between and within cells, has been implicated to play crucial roles in the regulation of the cardiovascular and nervous systems, and in other homeostatic mechanisms as well[2]. It is well known that NO regulates vascular permeability, inflammatory response, and immune system which is an essential prerequisite to implantation. Indeed, NOS activity and NOS protein increase in implantation sites compared with the interimplantation and nonimplantation regions in mouse and rat uteri[3].

The invasive event of implantation is dependent upon the synthesis and secretion of a varity of proteineases, of which one predominant family is matrix metalloproteinases (MMPs). NO is known to potentiate matrix degradation, which includes suppression of proteoglycan and collagen synthesis, and upregulation of metalloproteinase activity[4]. During the early implantation period, iNOS and MMP-2 enzymes are expressed proximally in the uterine epithelium and the level and activity of iNOS are increased several hours before the increase of MMP-2 activity in the uterus[5]. Moreover, inhibitors of NOS diminish MMP-2 activity, whereas NO donors enhance it in the isolated implantation rat uterine tissue[5]. These results indicate that NO can directly express MMP-2, an enzyme associated with cellular invasiveness, in the initial steps of a process, resulting in the breaching of uterine epithelia and endothelial integrity and the ultimate apposition of the maternal blood supply with embryo derived cells.

The major signaling pathway of NO is believed to be mediated by the activation of guanylate cyclase and the formation of cyclic guanosine monophosphate (cGMP)[6]. Although many aspects of MMP function during implantation have been elucidated, the relation of NO/cGMP and MMPs during implantation remains largely unknown.

The current study was designed to investigate the extent to which NO/cGMP participates in blastocyst adhesion, outgrowth and production of MMP-2 by using an *in vitro* culture system[7].

1 Materials and methods

(ⅰ) Reagents. Fibronectin (FN), gel and bovine serum albumin (BSA) were purchased from Sigma. RPMI 1640 and TRIzol were purchased from Gibco BRL. N^G-monomethyl-L-arginine, S-nitroso-N-acetylpenicillamine and 8-Br-cGMP were from Alexis. Oligo(dT) prime, reverse transcriptase AMV and Taq DNA polymerase were from Promega. GAPDH primer was a product of the Beijing Dingguo Biotechnology Development Center.

(ⅱ) Animals. Adult Kuming white mice were supplied by Institute of Zoology, the Chinese Academy of Sciences; they were housed at 25℃ in a controlled photoperiod (12 h light:12 h dark), allowed freedom to access water and food. Female mice were superovulated by intraperitonal injection of 10IU PMSG, followed by 10IU hCG 48 h later. After hCG injection, female mice were caged with one male of the same strain overnight. In the following morning, mice with vaginal plugs were designated as the first day of pregnancy (D1).

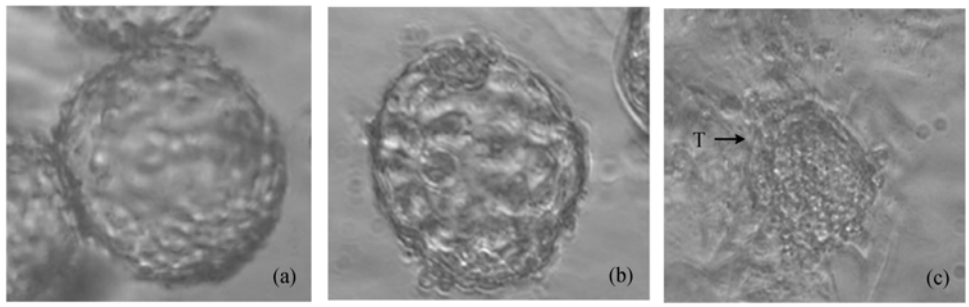

Fig. 1. Blastocyst cultured on the FN *in vitro* (original magnification ×150). (a) Blastocyst flushed out from uterus; (b) blastocyst with attachment; (c) blastocyst with outgrowth. T, trophoblast giant cells.

(iii) Embryo collection and culture. Blastocysts were flushed out from the uterus on D4 of pregnancy with RPMI 1640 and incubated in microdrops of RPMI 1640 medium complemented with 12.5 mmol/L $NaHCO_3$, 400I U/mL gentamycin sulfate and 0.5% BSA (5%CO_2, 37℃) for 24 h to allow the blastocyst to hatch from the zone pellucid. The culture plate was precoated with 10 uL fibronectin (1.0 mg/mL), microdrops (40 μL) used to culture the embryos which contained the following substances as different treat groups. Group 1 contained nothing as control; Group 2 contained 500 μmol/L L-NMMA; Group 3 contained 500 μmol/L L-NMMA and 100 μmol/L SNAP; and Group 4 contained 500 μmol/L L-NMMA and 20 μmol/L 8-Br-cGMP. Hatched well-developed blastocysts (Fig. 1(a)) were sorted and transferred to these microdrops (49—52/microdrop) and cultured (5% CO_2, 37℃) in a humidified chamber for 12, 24 and 36 h.

(iv) Observation of attachment and outgrowth of blastocysts. The adhesion and outgrowth of blastocysts were observed under the phase contrast microscopy (Olympus, USA). While the plates were shaken gently, blastocysts that stay at the same place were recorded as adhesion (Fig. 1(b)). After adhesion, the blastocysts that grow out primary macrotrophoblast cells were recorded as outgrowth (Fig. 1(c)). The ratio of blastocysts with adhesion or outgrowth vs. the total number of embryos was adopted to evaluate the implantation capacity of blastocysts *in vitro*[8].

(v) Reverse transcription-polymerase chain reaction (RT-PCR). The total RNA isolated from embryos cultured in RPMI 1640 was subjected to semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). RNA was reversely transcribed (RT) by oligo (dT) primer and AMV reverse transcriptase. PCR amplification was carried out with 5 μL RT product from embryo (approximately 20 ng RNA). The amplification protocol consisted of cycles at 94℃ for 5 min, followed by 22 cycles of at 94℃ for 30 s, 53℃ for 30 s and 72℃ for 30 s, and then by a final step at 72℃ for 10 min. Ten microliters of the PCR products were visualized under ultraviolet light on 1.5% agarose gels containing 1 μg/mL ethidium bromide. The concentrations of the signals for the mRNAs on the autoradiograms were calculated between the MMP-2 and GAPDH. GAPDH upper prime was 5′-GTG GGG CGC CCC AGG CAC CA-3′, GADPH downer prime was 5′-CTT CCT TAT TGT CAC GCA CGA TTT C-3′, the expected fragment size was 540 bp; MMP-2 upper prime was 5′-CAC CTA CAC CAA GAA CTT CC-3′ and MMP-2 downer prime was 5′-AAC ACA GCC TTC TCC TCC TG-3′; the expected fragment size was 325 bp.

(vi) Gelatin zymography. The presence of gelatinolytic MMPs in media was detected by gelatin zymography. The harvested 36 h culture media were standized according to the protein content of cell lysates measured using the method of Bradford[9]. Thus, 10—20 μL medium, equivalent to 6 μg protein of cell lysates, was loaded onto each lane for zymography. The medium was mixed in a ratio of 5∶1 (*v*∶*v*) with a sample buffer and then applied to gels for electrophoresis without boiling in 15% acrylamide gel co-polymerized with 1 mg/mL gelatin. After electrophoresis, the gels were washed at room temperature for 1 h in 2.5% Triton X-100, 50 mmol • L^{-1} Tris-HCl, at pH7.5, to remove SDS and incubated at 37℃ for 2—3 d in buffer (150 mmol • L^{-1} NaCl, 5 mmol • L^{-1} $CaCl_2$, and 50 mmol • L^{-1} Tris-HCl, pH 7.6). Thereafter, gels were stained for 60 min with 0.1% (*w*∶*v*) Coomassie Brilliant Blue R-250 in 30% (*v*∶*v*) isopropyl alcohol, 10% glacial acetic acid and destained in 10% (*v*∶*v*) methanol and 5% (*v*∶*v*) glacial acetic acid. Gelatinolytic activities were detected as clear bands on a uniformed blue background. Gels were photographed and the band intensities were quantified by densitometry of the negatives on a PDI protein plus DNA Imageware System (Huntington Station, NY, USA).

(vii) Statistical analysis. The data were presented as the means±SD. Difference was evaluated with the Student's *t*-test. Values of $P < 0.05$ were accepted as significant.

2 Result

(i) The effects of NO/cGMP on mouse blastocysts

adhesion and outgrowth. In the current study, it was found that there were significant differences in the percentage of blastocyst adhesion and outgrowth among different groups. L-NMMA alone could inhibit both the adhesion and outgrowth of blastocysts significantly compared to the control group ($P < 0.01$); however, SNAP, a NO donor, could rescue the inhibitory effect of L-NMMA. Similarly, the cGMP analogue, 8-Br-cGMP, could also reserve the inhibitory effect of L-NMMA on blastocyst adhesion and outgrowth (Figs. 2 and 3).

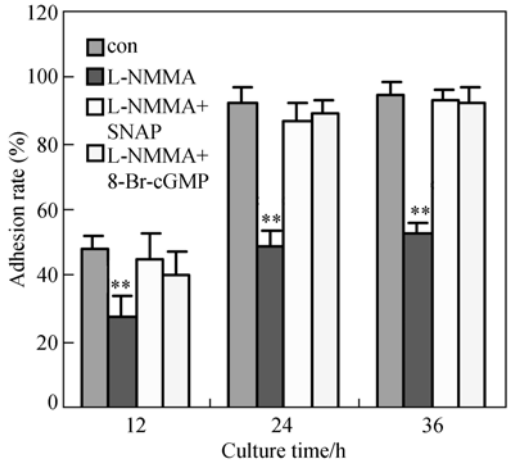

Fig. 2. Effect of NO/cGMP on adhesion of blastocysts. ** Compared with control group, $P < 0.01$.

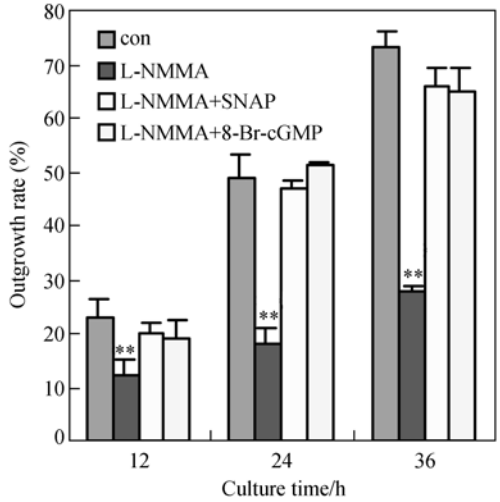

Fig. 3. Effect of NO/cGMP on outgrowth of blastocysts. ** Compared with control group, $P < 0.01$.

(ii) NO/cGMP pathway regulated the mRNA and protein expression of MMP-2. It was found that there was significant attenuation in the expression of the mRNA and protein of MMP-2 when the blastocysts were treated with L-NMMA alone. However, SNAP and 8-Br-cGMP reserved the inhibitory effect of L-NMMA on the expression of the mRNA and the production of MMP-2 protein (Figs. 4 and 5).

3 Discussion

Implantation of embryo into the lumenal epithelium of the endometrium is one of the most important steps in the establishment of intimate maternal-fetal connection and in the maintenance of a viable pregnancy. Successful implantation depends on invasive blastocyst, acceptant endometrium and their synchronization. Maternal steroids, prostaglandins, chronic gonadotropin, cytokines, adhesion molecules, proteinases and proteinase inhibitors are involved in this complex process. Here we present the first direct evidence that the NO/cGMP signaling pathway affects the mouse blastocyst adhesion and outgrowth *in vitro* and the results are consistent with the fact that NO participated in implantation *in vivo*[10].

It is reported that NO signaling pathway is rather complex and the knowledge of it is far from being complete. For example, much is yet to be done to make clear the impact of NO on cellular function, in which NO effects MAPK signal pathway[11] and NO regulated the meiotic maturation of mouse oocytes via cAMP[12]. One effect of low concentrations of NO is the activation of the enzyme soluble guanylate cyclase (soluble GC), which results in an elevation of intracellular cGMP. The effects of cGMP are mediated by binding to cGMP-dependent phosphodiesterases (PDEs), ion channels, or protein kinase G (PKG)[13,14]. Our study shows that cGMP analogue 8-Br-cGMP may rescue the inhibitory effects of L-NMMA on the adhesion, outgrowth and MMP-2 production of the mouse embryo.

Although the mechanisms that control MMP_S levels are often controlled by complex and reciprocal interactions between adjacent cell types, the understanding of the processes that control MMP_S expression has lagged behind the recognition of their significance. Choe et al. reported that the production of MMP-1 and -2 is regulated through the signaling pathway involving NO and that it can be downregulated by the NOS inhibitor[15]. Franchi et al. reported that *p53* mutation, which is frequently present in HNSCC, may raise angiogenesis and invasiveness related to the increased nitric oxide and MMPs production by tumor cells, ultimately contributing to tumor progression[16]. A number of previous studies have shown that NO derivatives can modulate MMPs activity in culture, for example, during metastasis progression of carcinosarcoma[17].

The regulation of MMPs occurs at different levels, including gene expression, processing of the inactive proenzymes by removal of a 10 kD fragment and by inhibition of the activity enzymes with their endogenous delineated as tissue inhibitors of matrix metalloproteinases[18]. Our study shows that exogenous NO/cGMP leads to an increase in the MMP-2 mRNA of blastocyst *in vitro*, accompanied by an increase in MMP-2 processing and activity, and the process is at least regulated by the NO/cGMP.

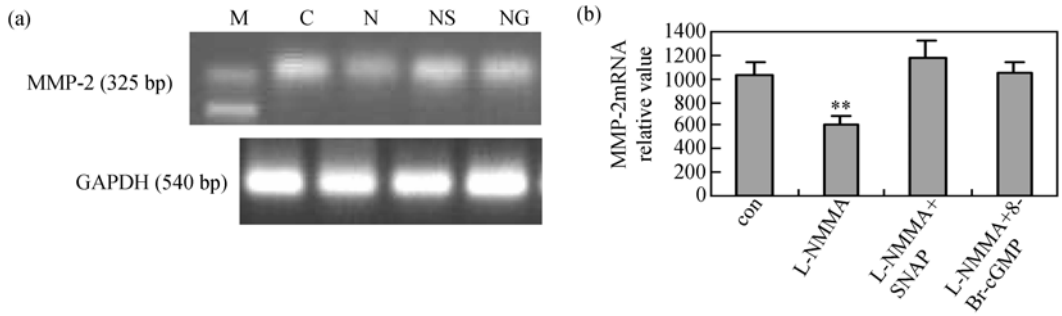

Fig. 4. Effect of NO/cGMP on MMP-2mRNA of blastocysts. (a) Results of RT-PCR for MMP-2mRNA. C, Control; N, L-NMMA; NS, L-NMMA+SNAP; NG, L-NMMA+8-Br-cGMP. (b) all data are the concentrated of bands of MMP-2mRNA by densitometry. Results are representative of three different experiments. ** Compared with control group, $P < 0.01$.

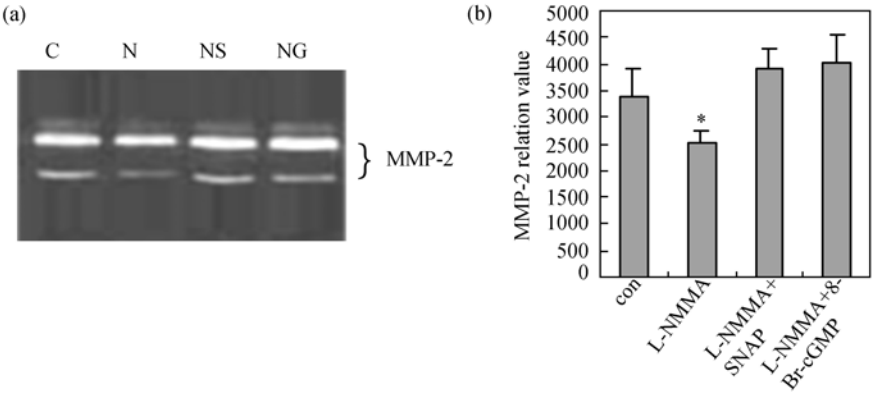

Fig. 5. Effect of NO/cGMP on MMP-2 protein production of blastocysts. (a) Results of gelatin zymography for MMP-2 production. C, Control; N, L-NMMA; NS, L-NMMA+SNAP; NG, L-NMMA+8-Br-cGMP. (b) All data are the concentrated sun of bands of MMP-2 production by densitometry. Results are representative of three different experiments. * Compared with control group, $P < 0.05$.

In conclusion, we provide the first evidence that cGMP signaling pathway was involved in the NO regulation of mouse embryo implantation and NO/cGMP might upregulate MMP-2 expression at the mRNA level *in vitro*.

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