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Nitric Oxide and Embryo Development

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Ⅰ. WHAT IS THE NITRIC OXIDE (NO)

NO has gained wide recognition through a number of diverse activities in physiology and pathology. This molecule, Science magazine's "molecule of the year 1992", is a key transducer of the vasodilator message from the endothelium to vascular cells. Furthermpre, it is a constituent in central and peripheral neuronal transmission, and participates in nonspecific immne response. As a radical, NO displays high reactivity in a large number of biological processes attributed to both beneficial and harmful reactions to the body tissues. The expression of a wide variety of NO effects is achieved through both coordinative interactions with metals and redox events. Reactions with oxygen (O_2), superoxide (O_2^-) and transition metals produce various NOx species, peroxinitrite (ONOO-) and metal-NO adducts, respectively. However, main NO signaling can be categorized by a cGMP-independent versus cGMP -dependent secondary pathways.

Observations made in 1916 suggested that human tissues synthesize NO as detected in the urine (Mitchell et al.), but this was not verified until the early 80's. It was subsequently shown that mammalian species produce NO_3^- and that the formation of this product is increased by endotoxin treatment (Tannebaum et al., 1978; Green et al., 1981). In 1985, evidence for induced nitrite and nitrate synthesis in murine macrophages was provide (Stuehr and Marletta, 1985). Subsequently, the role of NO in smooth muscle relaxation accounted for endothelium-derived relaxing factor activity (Furchgott and Zawadzki, 1980). Including these cells, it has been confirmed to date that a number of tissue cells can produce NO such as endothelial cells (Palmer et al., 1987), tumor cells (Amber et al., 1988), Kupffer cells (Billiar et al., 1989), chondrocytes (Knowles and Moncada, 1994), uterine tissue cells (Yallampalli et al., 1994), fibroblast (Lavnikova and Laskin, 1995), pancreatic islet cells (Welch and Sandler, 1992) and brain tissue cells (Bradt et al., 1990).

To produce NO, three types of NO synthase involve (Nathan and Xie, 1994); neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) NO synthase. nNOS was first identified in neuron and eNOS in endothelial cells. A cytokine-inducible iNOS expression has been found in various cell types receiving immunological or inflamatory stimuli. The activity of nNOS and eNOS depends on Ca^{2+} above the resting level, while iNOS is independent of Ca^{2+} to to some extent. The reaction catalyzed by NOS is the oxidation of l-arginine to produce citrulline and stoichiometric amounts of NO. All NOS isozymes are P450-like hemoproteins, contain binding sides for flavin adenine dinucleotide and flavin mononucleotide, and carry out a five electron oxidation of the substrate in the presence of tetrahydrobiopterin.

Ⅱ. WHAT TRIGGERS THE NO RESEARCH IN EMBRYOLOGY

Part of this study was supported by a grant from Korea Research Foundation (KRF-99-041-G00065).

Accidently, we found the possibility that NO involves embryo development in 1996. We did in vitro-fertilization (IVF) of bovine oocytes with epididymal semen of a pelvic fractured bull and the semen was totally contaminated with the whole blood. Surprisingly, the blastocyst formation of embryos derived from IVF was greatly increased more than 50% and this rate was retrospectively higher than that of our normal rate. Based on these results, we hypothesized three possibilities; 1) blood cells neutralizes oxygen toxicity to embryos, 2) blood cells secrete various embryotropic growth factors or 3) heme molecule in the blood involves NO action. Considering medium composition used in the experiment, the possibilities of oxigen detoxication and growth factor secretion were removed, since the medium contains a number of antioxidant and growth factors. To confirm the hypothesis on NO, we subsequently measured NO metabolites in used medium and found that there is a significant increase in NO metabolites accompanying increased culture duration. Through this observation, we established the hypothesis that NO affects embryo development *in vitro*.

Ⅲ. HOW NO AFFECTS EMBRYO DEVELOPMENT

The question how NO affects embryo development was arisen to confirm our hypothesis. Firstly, we made an experiment to add a spontaneous NO donor, sodium nitroprusside (SNP), to culture medium and embryos were cultuted in the medium after added SNP to culture medium at either 18 or 60 hours postinsemination. The addition of SNP significantly inhibited embryo development and this action appears as a peracute fashion. The inhibitory action of SNP was also shown when it added to the medium for culturing individual embryo. This even appeared at the concentrations of SNP lower than 40 μM.

In the next series of experiment, we measured the intracellular concentration of NO by ELISA. For this experiment, both developing and developmentally arrested embryos were collected at 48 and 144 hours postinsemination. Although there was no difference in intracytoplasmic NO concentration in the embryos collected at 48 hours postinsemination, arrested embryos at 144 hours postinsemination had significantly higher NO concentration than developing embryos. Such data clearly suggested that NO negatively affects the preimplantation development of bovine embryos cultured *in vitro*.

Ⅳ. HOW TO NEUTRALIZE THE TOXIC EFFECT OF NO

There are two ways to prevent the embryotoxic NO action; by inactivating secreted NO using NO scavengers or by blocking NO synthesis using NOS inhibitors. To find a way to inhibit NO action on embryo development, hemoglobin (Hb) and l-nitro-arginine-methylester (L-NAME) were used as a NO scavenger and a NOS inhibitor, respectively. In the first set of experiment, we evaluated the effect of Hb on embryo development. Bovine 1-cell embryos were cocultured with cumulus-granulosa cells and either 1 or 10 μg/mL Hb was added at the onset of culture. A significant increase in blastocyst formation was achieved by the addition of 1 μg/mL Hb compared with by no addition. Ten microgram/mL Hb also increased the blastocyst formation, but this increase was not as high as that observed after the addition of 1 μg/mL Hb. To examine the effect of cumulus-granulosa cells on Hb effect, Hb was used in a cumulus cell-free culture system and such usage did not increase the blatocyst formation of embryos.

It was possible that such results were affected by the quality of embryos collected from different

seasons. We then used Hb-free and Hb-containing media for embryo culture for 7 months from November 1996 to May 97. More than 1,600 embryos were used for this study and, regardless of the months, Hb-containing medium constantly yielded higher blastocyst formation rates than Hb-free medium. The model effect of this treatment was less than 0.00001 by ANOVA.

In the next set of experiment, we examined the effect of L-NAME on the development of bovine embryos cultured in Hb-containing medium. Either 1 or 1,000 nM L-NAME was added to the medium, but no significant increase in blastocyst formation was found. Although there was a limitation to interpret the action of a NOS inhibitor on embryo development, these data indicated that embryo development can be improved by the use of Hb, a NO scavenger. A NOS inhibitor is not effective under the presence of Hb in embryo culture system.

V. IS A NO SCAVENGING SYSTEM AVAILABLE FOR THE CULTURE OF EMBRYOS IN OTHER SPECIES?

All of the data shown in the above chapter is mainly came from the study using bovine embryos. Therefore, it is necessary to examine whether the inhibition of NO action is beneficial for supporting in vitro embryo development in other species. Subsequent study was designed to examine the effect of Hb on mouse embryo development and outbred "block" strain mouse (ICR) was employed. Through this experiment, we used a chemically defined preimplantation-1 medium (P-1) as a based medium. The addition of Hb to P-1 significantly stimulated the development of 1-cell embryos to the blastocyst stage, compared with no addition. Based on this information, modified P-1 was designed. It yielded higher blastocyst formation rate of more than 60% and improved the quality of the blastocysts, which was appeared as increased total blastomere and inner cell mass cell numbers. Accordingly, the NO scavenging system is available for mouse embryo culture, which improves *in vitro* development of ICR mouse 1-cell embryos.

Ⅵ. CONCLUSION

Studies on the role of NO in embryo development much contributed to developing a number of reproductive biotechnology. However, further study is required to elucidate the biological aspects of NO action in embryo development.

Ⅶ. ACKNOWLEDGMENTS

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요 약

산화질소와 수정란 발생

임 정 묵

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산소와 질소의 단순 화합물질인 산화질소 (nitric oxide)는 생체내의 다양한 작용을 야기하는 시그날 물질로 주목을 받고 있으며 이의 작용에 대한 연구가 광범위하게 진행되고 있다. 본 논문을 통하여 저자는 수정란 대사에 미치는 산화질소 작용규명이 지금까지 어떻게 진행되어 왔는지 소개하였으며 본 물질에 대한 최근 연구결과를 설명하였다. 일련의 실험결과 산화질소는 수정란의 초기발생 저해인자로 작용하며 수정란 배양 중의 산화질소작용 억제에 의하여 배반포 발생이 현저히 증가한다는 사실을 밝혀냈다. 참고로 본 논문내용은 Mol. Reprod. Dev. (1998), Theriogenology (1999) 및 Fertil. Steril. (in press)에 개재된 연구결과를 요약한 것이다.

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