# THE ROLE OF L-ARGININE IN CONTROL OF APOPTOSIS IN PREIMPLANTATION MOUSE EMBRYOS CULTURED IN HIGH GLUCOSE MEDIA

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## ABSTRACT

Maternal hyperglycemia causes delay in early stages of embryonic growth and development, higher incidence of congenital malformations and spontaneous miscarriage compared with those of non-diabetic conditions. High glucosis tratogenicity seems to be related to reduction of Nitric Oxide production (NO) in hyperglycemic condition. In order to test this hypothesis, 2-cell stage embryos of normal mice were cultured with high concentration of glucose (30mM) and different concentrations of L-arginine (5,10,20 mM) or L-NAME, an NO syntase (NOS) inhibitor. In the end of culture, blastocysts were stained by by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique and apoptotic cells were detected by using a Fluorescence microscope. Finally the amount of nitrite in the cultured media was assayed by Griess method.

The results indicated that high glucose reduces Nitric Oxide production by preimplantation embryos and increases apoptosis of embryonic cells, but 5-20mM of L-arginine significantly increases Nitric Oxide production and decreases apoptosis. On the contrary L-NAME significantly inhibits the development of pre-implantation embryos.

In conclusion, this study indicated that reduced nitric oxide production in high glucosis condition is a main factor for embryonic damage, and supplementation of high glucose media with L-arginine has an important role in prevention of high glucosis embryotoxicity.

**Key Words:** High glucose, Apoptosis, Nitric Oxide, Pre-implantation Embryo.

## INTRODUCTION

Diabetic pregnancy is associated with spontaneous abortions and growth disturbance and congenital malformations in the offspring (1-5).

The complexity of the intrauterine environment makes it difficult to identify the mechanisms leading to this embryopathy, however it has established that high concentrations of glucose in the uterine lumen of the diabetic females has a direct effect on pre-implantation embryos (6).

In vitro studies have indicated that the high glucose levels (17-30 mM) in culture media adversely affect the morphological development of pre-implantation mouse and rat embryos and increase apoptosis among the blastomers specially in inner cell mass (ICM), and cause a reduction in total cell number of blastocysts (7,8).

The mechanism of high-glucosis induced embryo toxicity is not clear properly. However according to the results of studies which performed on other type of cells (9,10), it is postulated that the derangement of the nitric oxide system may induce deleterious effects of high glucosis on embryonic cells.

Nitric oxide is produced when the various isoforms of enzyme NO synthase (NOS) catalyze the oxidation of L-arginine to L-citrolline. It has been suggested that NO has important physiological roles in a varity of reproductive processes such as follicular development, ovulation and spermatogenesis. Some investigations have indicated that various isoforms of NOS are expressed in the oocyte, early preimplantation embryo, and uterus during

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pregnancy, and NO production at this time has an important role on embryo development and implantation (11-13). Couge et al. for the first time demonstrated that NO is produced in preimplantation embryos and its production is required for normal embryonic development and preimplantation embryonic development is inhibited when NO production is impaired (11). Huei-Wen et al. and Kazuo et al. (12,14) also found that a moderate amount of NO production is essential for embryo development, but that excessive amount of NO generation might inhibit embryo development and thus lead to infertility. They indicated that physiological amounts of NO, which is secreted from embryos themselves, are required for normal embryonic development and NO production of embryos is finely regulated. Preimplantation embryos may be highly sensitive to alteration of NO levels, and even subtle changes of NO levels could interfere with embryo development. According to the results of some studies, hyperglycemic condition reduces nitric oxide production in cells in vivo and in vitro (9,15,16,17). One possibility of impaired NO synthesis in hyperglycemic condition is a decreased availability of L-arginine, the substrate necessary for NO production (17).

L-arginine supplementation has indeed been shown to improve impaired NO activity in diabetic animal models (10). According to these data it is postulated that high glucosis may induces its embryo toxicity through derangement of the nitric oxide system.

There are no reports about the effects of hyperglycemia on NO production by preimplantation embryos and its relation with embryonic damages. Therefore, in the present study we evaluated the NO production by preimplantation mouse embryos during culture in high glucose medium with or without supplementation of 5,10,20 mM L-arginine and its effects on apoptosis in the embryonic cells.

## MATERIALS AND METHODS
### Culture mediums
Media used for embryo culture were based on modified human tubal fluid medium (HTF) (18) with different concentration of glucose and L-arginine or L-NAME (an antagonist of L-arginine and inhibitors of NOS), as follow:

1-HTF (containing 2.87 mM glucose for control group).
2- HTF-HG (High glucose medium containing 30 mM glucose).
3- HG-5 LA (High glucose medium with 5 mM of L-arginine).
4- HG-10 LA (High glucose medium with 10 mM of L-arginine).

5- HG-20 LA (High glucose medium with 20 mM of L-arginine).
6- HG- L-NAME (High glucose medium with 1 mM L-NAME).
7- HTF - L-NAME (High glucose medium with 1 mM L-NAME).

All of these media supplemented with 4 mg/ml Bovine serum albumin (sigma) and equilibrated 24 hours before culture in incubator at $37^{\circ}C$ in 5% $CO_2$ in air.

### Embryo collection and culture
2- Cell stage embryos obtained from 5-6 week-old NMRI female mice super ovulated with 10 IU of human menopausal gonadoterophin (HMG) followed 48h later by 10 IU of human chorionic gonadotrophin (HCG) given by intraperitoneal injection.

The female mice mated with adult male of the same strain immediately after the injection of HCG and checked for mating the following morning.

The mated female mice were killed by cervical dislocation 48h after the HCG injection, their oviducts were removed and the 2-cell embryos were flushed from oviducts. The flushing medium was a HEPES-Buffered bicarbonated media (HTF-HEPES).

2- Cell stage embryos were pooled, washed in HEPES-HTF and placed randomly in groups of 25-30 embryos in the different test media at $37^{\circ}C$ in 5% $CO_2$ in air and cultured for 96 hours. Only morphologically normal embryos were used and the experiment was repeated 5 times and finally 125-150 embryos were examined in each group.

### Apoptosis Detection
To analyze the status of chromatin in embryos, we used a combined technique for simultaneous nuclear staining and TUNEL by modification of the procedures of Brison and Scholtz (19).

Briefly, after 96 hours culture, embryos were fixed overnight at $4^{\circ}C$ in 3.7% Para formaldehyde diluted in PBS. Following fixation, they were washed 4 times in PBS/PVP, and permeabilized in PBS containing 0.1% Triton-X100 for 1 hour and incubated in flurescein conjugated- dutp and TdT (TUNEL reagents), (Boehringer mannhien, Tokyo, Japan) for 1 hour in an incubator at $37^{\circ}C$ and 5% $CO_2$ in air. Positive controls were incubated in deoxyribonucleare I (Dnase I, 50 Mg/ml; sigma) for 20 min at $37^{\circ}C$ and 5% CO2 in air and negative controls were incubated in fluorescein – dutp in the absence of TdT.

After TUNEL, the embryos were washed 3 times in PBS/PVP and incubated in RNAse (50 mg/ml,

sigma) for 60 min at room temperature then incubated in PBS containing propidium iodide (PI, 50 μg/ml) for 1 hour at room temperature to label all nuclei.

After incubation, they were washed 4 times in PBS/PVP and mounted on a glass slide and examined in whole mounts under a fluorescence microscope with an excitation filter of 460-490 nm and a barrier filter of 514 nm.

The number of total cells and nuclei labeled by TUNEL was counted. Then for each blastocyst the incidence of TUNEL positive nuclei was calculated as percentage of the total cell number.

*Nitrite Assays*

NO concentration in the collected culture media was determined indirectly by measurement of nitrite ($NO_2$). Nitrite is stable degradation products of NO and is considered to be a reliable indicator of NO production in cell culture. NO secretion was detected by a micro titer plate Greiss assay (11). Briefly, Collected culture media were reacted with equal volumes of Greiss reagent (0.5% sulfanilamide, 0.05%naphthalene diamine dihydrochloride in 2.5% orthophosphoric acide), noncultured medium, and cultured media. All sample were run in duplicate. Assays were performed at room temperature for 10 min. Microtiter plates were read at 550 nm using ELISA photometer.

Linear regression was used to determine NO concentration from the standard curve of $NaNO_2$.

*Statistical analysis*

Differences among the means of total cell number and apoptotic cell indices were compared by one-way analysis of variance coupled with Dunett 3 and Tukey Post Hoch tests using SPSS version 10.

## RESULTS

As shown in Figure (1) nitrite amount in cultured high glucose medium significantly decreased compared control medium (P<0/0001). Also this figure shows that adding L-arginine to the high glucose medium significantly increases nitrite concentration in cultured treatment media in a dose-dependent manner. In contrast, Adding L-NAME to control or high glucose media completely inhibits Nitric Oxide production.

The mean of total cell number in control group is $129.14 \pm 7.4$, but glucose at concentrations of 30mM reduces the total cell number ($85.2 \pm 8$) and there is a significant difference between the HG and control groups (p<0.001)(Fig.2). On the other hand, adding L-arginine to the high glucose medium increased the total cell number in a dose-dependent manner (Fig 2). There are significant

differences between the HG and treatment groups (P < 0.001). However, significant difference was also noted between control and treatment groups (P < 0.001). Embryos from the HG-LNAME and HTF-LNAME groups were fragmented in the pre-morula stage. L-NAME significantly inhibited the embryo development to the blastocyst stage thus these groups omitted from cell counting program.
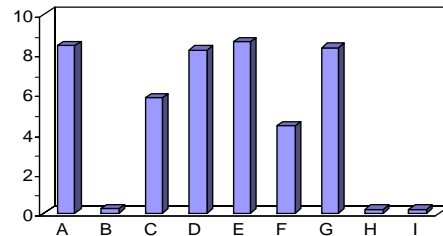


**Figure 1** Nitrite amount in cultured high glucose medium significantly decreased compared control medium (P<0.0001). Also this figure shows that adding L-arginine to the high glucose medium significantly increases nitrite concentration in cultured treatment media in a dose-dependent manner. In contrast, Adding L-NAME to control or high glucose media completely inhibits Nitric Oxide production (A: Control, B: HG, C: HG-5LA, D: HG-10LA, E: HG-20LA, F: HG-ASA, G: HG+5LA+ASA , H: Con-LNAME, I: HG-LNAME).
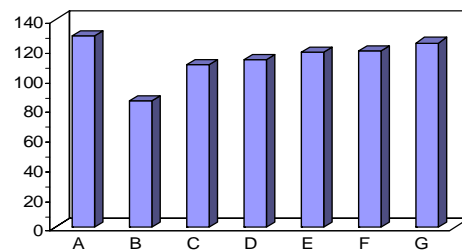


**Figure 2** The mean of total cell number. There is a significant difference between the HG and control groups (p<0.001) On the other hand; adding L-arginine to the high glucose medium increased the total cell number in a dose-dependent manner. However there are significant differences between the HG and treatment groups (P < 0.001), (A: Control, B: HG, C: HG-5LA, D: HG-10LA, E: HG-20LA, F: HG-ASA, G: HG+5LA)

As shown in figures (3) and (4) the proportion of apoptotic cells in embryos cultured in HG group was significantly higher than those cultured in control (P<0.001) (Fig5), but the proportion of apoptotic cells in embryos cultured in treatment groups (Fig 6) was significantly lower than embryos cultured in HG group (P<0/001), However, it was higher than those cultured in control (P<0.001).

## DISCUSSION

Today it is established that Nitric Oxide production by female reproductive system and

preimplantation embryos has an important role in embryonic development and implantation, such that suppressed NO production cause impaired embryonic development and implantation (11-13). In the other hand some studies have shown that hyperglycemic condition inhibits the NO production. Decreased serum levels of L-arginine in hyperglycemic conditions are especially considered as one of the main disturbing factors in the embryonic growth and cell death.
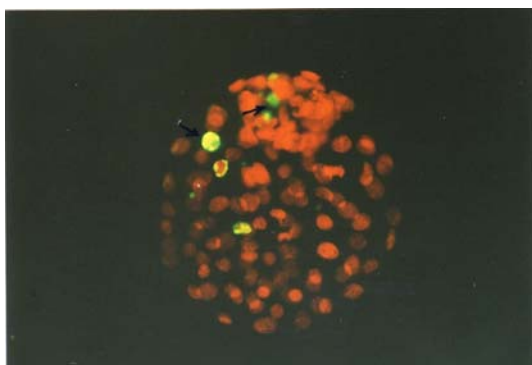


**Figure 3** a blastocyst from control group after TUNEL. The fragmented nuclei that labelled by TUNEL are shown as bright yellow and the normal nuclei are shown in red.
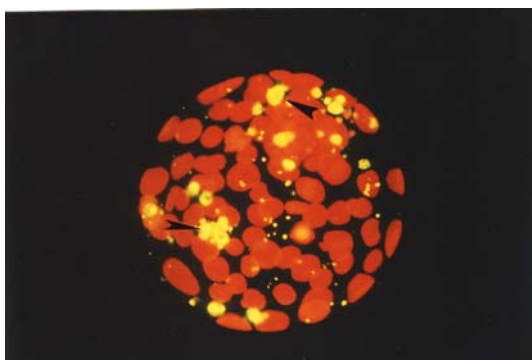


**Figure 4** a blast cyst from HG group. The proportion of apoptotic (bright yellow) nuclei is significantly higher than control.There are significant differences between the HG and control groups (P < 0.001).

Some investigators have shown that culture of cells in high glucose media inhibits NO production and provokes oxidative stress with subsequent increased production of reactive oxygen (ROS) (10,20). NO is an important factor which protects the cell from hazardous effects of ROS and there is always a critical balance between NO and ROS. This equilibrium might indeed have a crucial role in cell cycle and its life (21-23).
Several reports revealed the role of ROS in apoptosis of blastomers (24,25). Nitric Oxide is

considered an important factor for suppressing of ROS production and following cell death (22). ROS increasing and NO reduction are shown in diabetic mellitus (15-17). Low NO production in diabetic condition might be resulted from low L-arginine availability, because L-arginine uptake by cells is increased in this case (17).
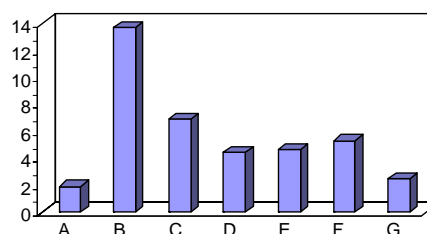


**Figure 5** the proportion of apoptotic cells in embryos cultured in HG group was significantly higher than those cultured in control (P<0.001), but the proportion of apoptotic cells in embryos cultured in treatment groups was significantly lower than embryos cultured in HG group (P<0.001), However, it was higher than those cultured in control (P<0.001), (A: Control, B: HG, C: HG-5LA, D: HG-10LA, E: HG-20LA, F: HG-ASA, G: HG+5LA).

In this study the effects of L-arginne as substrate of Nitric Oxide, and its potential protective effects on high glucosis induced apoptosis in pre-implantation mouse embryos were assessed. This study such as previous studies (6,7,8) indicates that total cell number in blastocysts cultured in high glucosis condition was decreased over 30% compared with the control group and high glucose medium raises the apoptosis index in blastomeres over 7 folds compared with the control group.
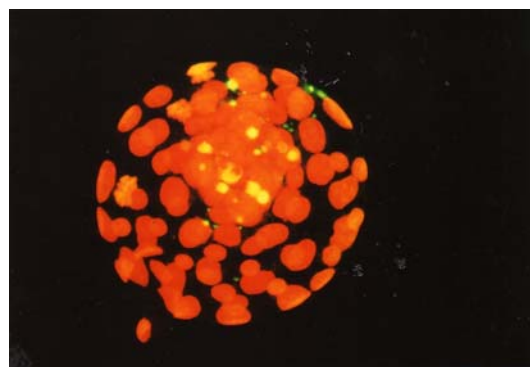


**Figure 6** a blastocyst from HG-10L-arginine. The proportions of apoptotic (bright yellow) nuclei are lower than HG. There are significant differences between the HG and HG-10L-arginine groups (P < 0.001).
Adding L-arginine to the high glucosis medium in a dose dependent manner decreased this index. However, there was also a significant difference between the apoptosis index of embryos cultured

in high glucose media containing L-arginine compared with control. These findings indicate that L-arginine could not be overcome to hazardous effects of high glucosis completely. This study, for the first time, indicates that reduced nitric oxide production in high glucosis condition is a main factor for embryonic damage, and supplementation of high glucose media with L-arginine has an important role in prevention of high glucosis embryotoxicity.

Based on these results, we suggest that dietary supplementation with L-arginine to the mother may prevents embryonic malformation in diabetic pregnancy by inhibition of oxidative stress generation. To establish a protocol for this proposes, it is necessary more information obtained from other in vitro and in vivo studies.

In conclusion, the addition of L-arginine to the high glucosis medium decreased the deleterious effects of the high glucosis on the pre-implantation embryos and helped them to maintain their developmental capacity.

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