Ascorbic Acid Diminishes Developmental Damages Induced by High Glucose in Mouse Pre-implantation Embryos *in vitro*

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Received 16 Sep 2001; accepted 14 Nov 2001

Summary

Diabetic teratogenicity seems that to be related to embryonic oxidative stress and the extent of the embryonic damage can apparently be reduced by antioxidants. In this study, 2-cell stage embryos of normal mice were cultured for 96 h with a high concentration of glucose (30 mM) in the absence or presence of 1 mM L-ascorbic acid (LAA). In the end of culture, the morphology of embryos was assessed with the aid of inverted phase-contrast microscope, and also cell apoptosis was studies by TUNEL (TdT-mediated conjugated dupt nick end-labeling) technique. Result: High glucosis alone decreased the blastosyst formation and increased the incidence of embryo fragmentation and cell apoptosis. With LAA, the incidence of high glucosis induced embryo fragmentation and cell apoptosis was decreased, on the contrary the incidence of blastocyst formation was increased.

Key words: high glucose, apoptosis, ascorbic acid, pre-implantation embryo, mouse, antioxidant

Introduction

Maternal diabetes adversely affects pre-implantation embryonic development in rodent models of both chemically induced and spontaneous diabetes (Pamfer *et al*

1990, 1995,1997, Verchval *et al* 1990, Hertogh *et al* 1991, Richard & Ioga 1996). It has been postulated that the diabetic embryotoxicity early in pregnancy may be a contributing factor to fetal complications known to occur later in diabetic pregnancy (Verchval *et al* 1990). Studies have reported an increase in fragmented embryos and a reduction in the number of cells of blastocysts, which recovered from diabetic rates (Pamfer *et al* 1990, Verchval *et al* 1990, Hertogh *et al* 1991). Some of these embryos were morphologically characterized as having cellular 'blebbing' nuclear condensation and an over expression of clustering indicative of apoptosis (Pamfer *et al* 1990,1997, Verchval *et al* 1990). 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 (Pamfer *et al* 1997).

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 (Moley & Maggie 1998a,b) and rat (Pamfer *et al* 1997) embryos, increase fragmented embryos and apoptotic cells and cause a reduction in total cell number of blastocysts. The role of apoptosis associated signaling pathways in this glucose embryotoxicity is unknown however, high glucose tratogenicity seems to be related to embryonic oxidative stress (Ornoy & Kimayagorov 1996, Salas *et al* 1998).

Some studies have indicated that reactive oxygen species (ROS) has an important role in diabetic-induced embryo toxicity in pre-implantation mouse embryos (Ornoy & Kimayagorov 1996) and pre-implantation embryos cultured in diabetic serum had a lower concentration of antioxidants than embryos cultured in non-diabetic serum (Zhonyhue & Zhi 1993, Yang *et al* 1998). Some evidences obtained over the past few years indicate that, in some cases, the generation of ROS is an important event during the course of apoptosis and the generation of oxidative stress is a common requirement for cell death (Salas *et al* 1998). Because of other evidences the concentration of low-molecular-weight antioxidant (LMWA) such as ascorbic acid (vitamin C) in cells decreased under diabetic culture conditions (Zaken *et al* 2001). Ascorbic acid is a major intracellular antioxidant and has an important role in

protection of cells against damages caused by ROS (Brennan *et al* 2000). It seems that high glucosis may induce embryo toxicity in pre-implantation embryos through derangement of the antioxidant defense mechanism (Zhonyhue & Chenzhi 1993, Xiaolin *et al* 1995,1998), so prevention of ROS damage is key for embryo development in diabetic or high glucose condition (Wetzel & Eriksson 1998). Based on these evidences, in this study, the effects of ascorbic acid as an exogenous antioxidant on high glucosis tratogenicity were examined using mouse pre-implantation embryo culture.

Materials and Methods

Culture Media. Media used for embryo culture were based on modified human tubal fluid medium (HTF) developed by Quinn *et al* (1985) with different concentration of glucose and ascorbic acid, as follow:

1) HTF (containing 2.87 mM glucose as a control group).

2) HTF-LAA (containing 2.87 mM glucose and 1mM ascorbic acid to control of toxicity of ascorbic acid).

3) HTF-HG (High glucose medium containing 30 mM glucose).

4) HTF-HG-LAA (High glucose medium with 1mM of ascorbic acid).

All of these media supplemented with 4 mg/ml bovin serum albomin (Sigma) and equilibrated 24 h before culture in incubator at 37°C in 5%. CO_2.

Embryo collection and culture. 2-Cell stage embryos obtained from 5-6 week-old B6C3F1 female mice super ovulated with 10IU of pregnant mare serum gonadotrophin (PMSG) followed 48h later by 10IU 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 HEPES-buffered bicarbonated medium (HTF-HEPES). 2-cell embryos were pooled, washed in HTF-

HEPES and placed randomly in groups of 15-20 in a drop of the different test media under light mineral oil (Sigma) at 37°C in 5% CO_2 and cultured for 96 h.

Assessment of morphology. Embryo morphology was assessed by use of phase-contrast inverted microscope 96 h after culture and the number of blastocysts and fragmented embryos was recorded.

Assessment of apoptotic cell death by TUNEL. To analysis the status of chromatin in embryos, we used a combined technique for simultaneous nuclear staining and TUNEL by modification of the procedures of Brison (Brison & Seholtz 1997, Brison & Burne 1999). Briefly, embryos were washed 4 times in PBS containing 1 mg/ml polyvinylpyrrolidone (PVP) and fixed overnight at 4°C in 3.7% ρ-formaldehyde diluted in PBS. The embryos were always processed with the Zona pellucida intact to preserve embryo morphology. Following fixation, they were washed 4 times in PBS/PVP, and permeabilized in PBS containing 0.1% Triton-X100 for 1h. Then they were washed 3 times in PBS/PVP and incubated in flurescein conjugated-dutp and TdT (TUNEL reagents), (Boehringer mannhien, Tokyo, Japan) for 1h in an incubator at 37°C and 5% CO_2. Positive controls were incubated in deoxyribonucleare I (DNase I; 50Mg/ml; Sigma) for 20min at 37°C and 5% CO_2 and washed twice in PBS/PVP before TUNEL. 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 (50mg/ml, Sigma) for 60min at room temperature. Then the embryos were washed 3 times in PBS/PVP and incubated in PBS containing propidium iodide (PI, 50mg/ml) for 1h at room temperature to label all nuclei. After incubation, they were washed 4 times in PBS/PVP and treated with 5%w/v of DABECO (1,4-diazabicyclo-2,2,2-octate; Sigma, Tokyo) in 90% glycerol:10% 0.2M Na_2HPO_3 to block the bleaching effect of fluorescence. They were then mounted on a glass slide and examined in whole mounts under a fluorescence microscope with an excitation filter of 460-490nm and a barrier filter of 514nm. The number of total cells and nuclei labeled by TUNEL were counted and for each blastocyst the incidence of TUNEL positive nuclei (T-index) was calculated as percentage of the total cell number.

Statistical analysis. Differences among the percentage of embryos developing in culture and differences in blastocyst cell number and apoptotic cell indices were compared by one-way analysis of variance coupled with Fisher test (Stat view 4.5).

Results

The *in vitro* development of 2-cell mouse embryos after 96h culture in HTF, HTF-LAA, HTF-HG and HTF-HG-LAA is summarized in table 1.

Table 1. *2-cell stage embryo development after 96h culture in HTF, HTF-LAA, HG and HG-LAA media*

Media	Total number of 2-cells embryo	Total number of blastocyst (% blastocyst±SD)	Total number of framgme embryos (%fragment±SD)
Control (HTF)	172	162 (%96.36±3.64)	10 (5.63±3.64)
HTF-LAA	178	168 (%94.54±4.56)	10 (5.45±4.56)
HG	176	83 (%47.14±3.63)	93 (52.85±3.63)
HG-LAA	183	132 (%71.8±10.16)	51 (28.19±10.16)

*The total number and percent±SD of blastocyst and fragmented embryos found from 10 times repeated experiments

The normal blastocyst and fragmented mouse embryos, and TUNEL analysis are shown in figures 1 and 2 respectively.

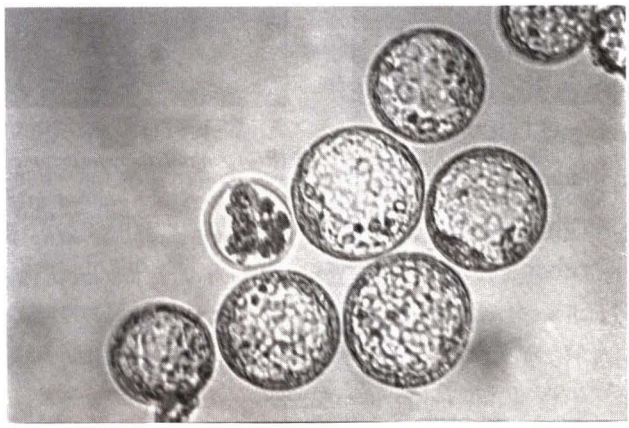
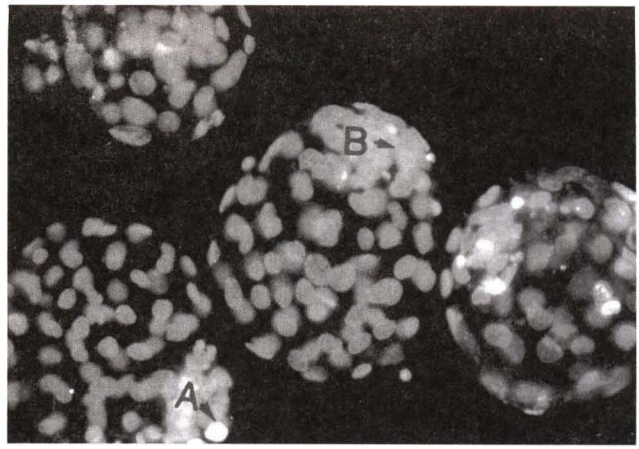


Figure 1. *Normal blastocyst (B) and fragmented (F) mouse embryos after 96h culture*



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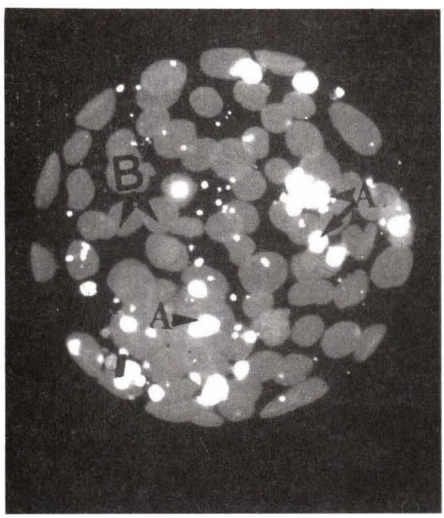

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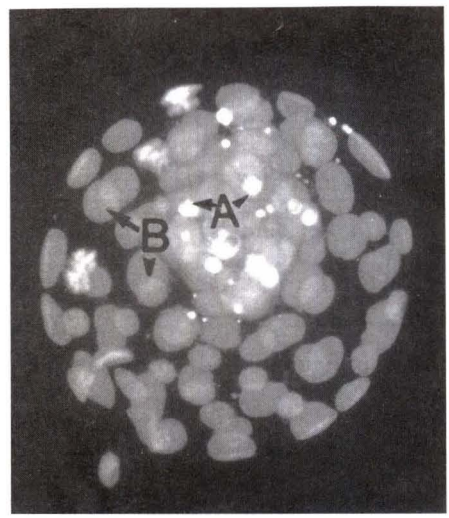

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Figure 2. *Combined nuclear propidium iodide (PI)/terminal transferase-mediated DNA end labeling (TUNEL) analysis. The nuclei in normal blastomers are stained in red (arrow B) (PI alone). DNA fragmentation in condensed chromatin is extensive as observed by an intense TUNEL signal in bright yellow as a result of the superimposition of red and green (arrow A). Blastcyst cultured in high glucose (b) compared with those cultured in control HTF (a) shows increased TUNEL positive nuclei. In blastocyst cultured in high glucose medium with 1mM L-ascorbic acid (c), apoptotic TUNEL positive nuclei was decreased and there is a significant differences between this group with high glucose (P=0.000) and control (P<0.001)*

Of the embryos in the control group 94.5 % had developed to the blastocyst or more advanced stages. The developmental rates of embryos in HTF-HG group were significantly lower (P<0.0001) than those of control embryos (Fig 3).

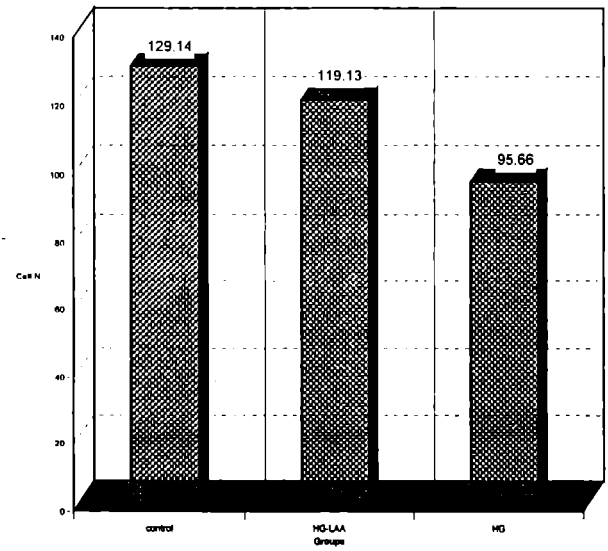

Figure 3. *The mean of total cell number in blastocysts, there are significant differences between control and HG (P<0.0001), control and HG-LAA (P<0.005) and HG with HG-LAA (P<0.001) groups*

It was found that supplementing the high glucose medium (HTF-HG) with vitamin C significantly increased blastocyst formation in compared to HTF-HG group (P<0.001), however there was a significant difference between the proportions of blastocyst in control and HTF-HG-LAA groups (P<0.001). Degenerated embryos were defined as those, which had a 100% blastomer fragmentation or pichnotic nucleus. After 96h culture, the proportion of degenerated embryos in the control group was 5.5% and in the HTF-HG group was significantly higher (P<0.0001). The percentage of degenerated embryos was significantly decreased in HTF-HG-LAA group compared with the HTF-HG (P<0.01) and control (P<0.001) groups. Embryos cultured in HTF had significantly more cells than those cultured in HTF-HG (P=0.000) and HTF-HG-LAA (P<0.024), but the total cell number in HTF-HG-LAA group significantly was higher than HTF-HG group (P=0.000).

There was differences between the proportion of apoptotic cells (Fig 4) per embryos culture in HTF-C and HTF-HG (P=0.000). The proportion of apoptotic cells in embryos cultured in HTF-HG-LAA groups was significantly lower than embryos cultured in HTF-HG (P<0.0001), but it was higher than those cultured in HTF (P<0.001).

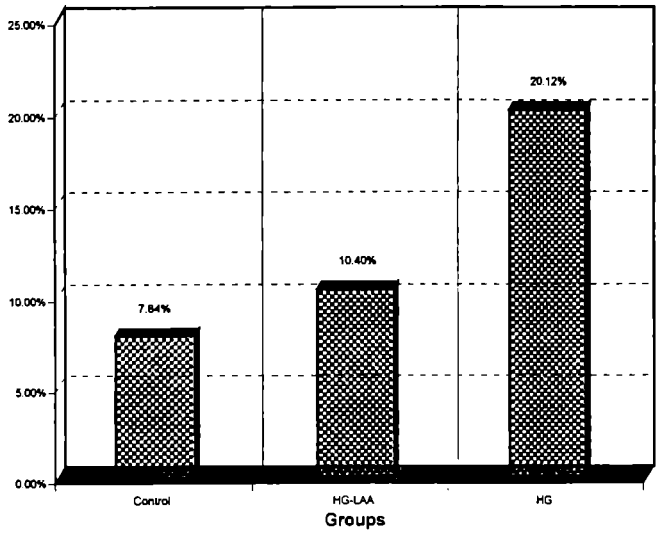

Figure 4. *Apoptosis index. There are significant differences between control and HG (P<0.0001), Hg and HG-LAA (P<0.0001) and control with HG-LAA (P<0.001) groups*

Discussion

Previous studies have indicated that production of ROS by embryonic mitochondria increases under diabetic condition (Salas *et al* 1998, Zaken *et al* 2001, Haedler *et al* 1996, Du & Sui 1998). And also, it is reported that in the embryos cultured in media contains high levels of sera from diabetic patients antioxidant defense mechanism was disturbed (Sabina *et al* 1995, Ornoy & Kimayagorov 1996, Pamfer *et al* 1997b). It seems that disturbed embryonic antioxidant defense mechanism may play a major role in diabetes-induced apoptosis and embryos fragmentation. It was found a high rate of congenital anomalies decreased growth and protein content and a decreased in total activity of both superoxide dismutase (SOD) and catalase (CAT) under diabetic conditions, as compared with controls (Ornoy & Kimayagorov 1996). HPLC studies

(Okuda *et al* 1991, Brennan *et al* 2000) have demonstrated a decrease in vitamin C and in vitamin E, low-molecular-weight antioxidants, (LMWA) under diabetic culture condition and also it is suggested that ROS are implicated in apoptosis of early embryos and prevention of ROS damage is important for embryo development (Salas *et al* 1998). However, the mechanism of antioxidant defense in early embryos is virtually unknown.

In this study the effects of ascorbic acid as an exogenous antioxidant, and its potential protective effects on high glucosis induced embryo malformation and DNA damage in pre-implantation mouse embryos was assessed. Mouse embryos at 2-cell stage were cultured for 96 h in the HTF medium with different concentration of glucose (3.4mm and 30mm) and 0.1mM LAA and in another group the embryos were cultured in HTF medium containing 30mm glucose which supplemented with 1mM LAA. High glucosis alone increased the incidence of embryo fragmentation and the development of the embryos was delayed when culture in high glucose medium. These significant differences in blastocyst formation and embryo fragmentation among the control and high glucose groups are similar to the values observed in previous studies (Moley & Maggie1998, Pamfer 1990,1997).

This study such as previous studies (Pamfer *et al* 1990, Hertoge *et al* 1991, Moley & Chi 1998) indicates that total cell number in blastocysts cultured in high glucosis condition was decreased and incidence of apoptotic cell in each blastocyst significantly increased compared with control blastocysts. We have shown that supplementation with LAA decreased significantly high glucose induced embryo and DNA damage, but it did not return to normal level. However, there was a significant difference between embryos in HG-LAA group in compared with control, it seems that the addition of vitamin C to the high glucosis medium decreased the deleterious effects of the high glucosis on the pre-implantation embryos. These supplementation regimens may be used to limit the possible adverse effects of ROS on pre-implantation *in vitro*, and also help to maintain their developmental capacity.

This study indicates that high glucosis has a direct effect on embryonic development. DNA damage may be occurred through production of ROS, distortion

of embryonic antioxidant defense mechanism and depletion of the LMWA such as vitamins C. The addition of this vitamin reduces the embryonic antioxidant defense mechanism and decreases the damages caused by the high glucose condition. Based on these results, we suggest that dietary supplementation with antioxidant to the mother may prevents embryonic malformation in diabetic pregnancy by inhibition of oxidative stress generation. To establish a protocol for this proposes we need more information obtained from other *in vitro* and *in vivo* studies.

Acknowledgments

Department of obstetrics and Gynecology, School of Medicine, Yamagata University, Yamagata, Japan supported this work. The support of Professor Hirohisa Kurachi (Chairman) and the technical assistance of Dr. Hidakazu Saito are greatly appreciated.

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