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Methylglyoxal has injurious effects on maturation of mouse oocytes, fertilization, and fetal development, via apoptosis

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ABSTRACT

Methylglyoxal (MG) is a metabolite of glucose. The serum MG level is increased in diabetic patients, and MG is implicated in diabetic complications related to embryonic development injury. We previously reported cytotoxic effects of MG on mouse embryonic stem cells and blastocysts, and a further association with defects in subsequent development. Here, we further investigate the effects of MG on oocyte maturation and subsequent pre- and post-implantation development, both *in vitro* and *in vivo*. Notably, MG induced a significant reduction in the rate of oocyte maturation, fertilization, and *in vitro* embryonic development. Treatment of oocytes with MG during *in vitro* maturation (IVM) led to increased resorption of post-implantation embryos and decreased fetal weight. Experiments with an *in vivo* mouse model disclosed that consumption of drinking water containing 10–20 μM MG led to decreased oocyte maturation and *in vitro* fertilization, as well as early embryonic developmental injury. Finally, pretreatment with a caspase-3-specific inhibitor effectively prevented MG-triggered injury effects, suggesting that embryo impairment by MG occurs via a caspase-dependent apoptotic process.

1. Introduction

Methylglyoxal (MG) is a metabolic product of glucose. Under hyperglycemic conditions, MG is formed from triose phosphates or acetol. MG serves as a precursor of advanced glycation end-products (AGEs) (Ahmed et al., 1997; Phillips and Thornalley, 1993). The serum concentration of MG or MG-derived AGEs in diabetic patients is significantly higher than that in non-diabetic individuals (Kilhovd et al., 2003; Lapolla et al., 2003). A number of studies suggest that diabetes causes impairment of cognitive processes via a mechanism involving both oxidative stress and AGE formation (Gerozissis, 2003; Messier and Gagnon, 1996). Thus, high MG levels and subsequent cytotoxicity may be at least partially responsible for diabetes-related impairment of cognitive function.

The cytotoxicity of MG is attributed to an ability to trigger apoptosis (Kang et al., 1996; Okado et al., 1996). In addition, MG-induced apoptosis occurs through mitochondrial-dependent processes (Du et al., 2000; Hsuuw et al., 2005). Earlier studies by Hsuuw et al. (2005) showed that MG elicits cell apoptotic biochemical changes, such as DNA fragmentation, caspase-3 activation, cleavage of PARP, mitochondrial cytochrome *c* release, and JNK activation, in mouse embryonic stem cells (ESC-B5). Moreover, MG induces apoptosis in mouse blastocysts (Hsuuw et al., 2005). MG-induced embryonic stem cell apoptosis and embryonic development injury is effectively prevented by ROS scavengers, implying a critical role for ROS in this process (Hsuuw et al., 2005). Several chemical and physical treatments that induce apoptosis stimulate oxidative stress via generation of reactive oxygen species (ROS) (Halliwell and Gutteridge, 1990; Hsuuw et al., 2005; Pathak and Khandelwal, 2006; Yan et al., 2006), implying a close relationship between oxidative stress and apoptosis.

Oocyte viability is affected by the microenvironment during growth and maturation. Heat stress, oxygen concentration, and glucose content are key determinants of oocyte viability (Banwell et al., 2007; de Castro and Hansen, 2007; Sartori et al., 2002). A number of researchers have investigated the influence of environmental biological toxins on oocyte maturation *in vivo* and *in vitro*. During normal embryogenesis, apoptosis (a unique morphological pattern of cell death) functions to remove abnormal or redundant cells in preimplantation embryos (Hardy, 1997; Hardy et al., 2003). However, apoptotic processes do not occur prior to the blastocyst stage during normal mouse embryonic development (Byrne et al., 1999), and induction of cell death during oocyte maturation and early stages of embryogenesis (*i.e.*, via exposure to a teratogen) leads to embryonic developmental injury (Banwell et al., 2007; Chan, 2006, 2009; Hsuuw et al., 2005; Shiao and Chan, 2009). Previous studies by our group demonstrated that MG promotes cell apoptosis and developmental injury in blastocyst stage embryos which

Abbreviations: MG, methylglyoxal; COCs, cumulus–oocyte complexes; IVM, *in vitro* maturation; IVF, *in vitro* fertilization; ICM, inner cell mass; TE, trophectoderm.

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development from zygote for 4 days (Hsuuw et al., 2005). However, the influence of MG on early stage embryogenesis, such as oocyte maturation, fertilization, and sequential embryo development from zygotes is currently unclear. Here, we further investigate whether MG has a hazardous effect on oocyte development by incubating oocytes with the compound for 24 h and comparing sequential development with that of oocytes under MG-free conditions. We aimed to determine whether short-term exposure to MG at the oocyte stage had long-term injurious impacts on embryo development. Our results clearly demonstrate that MG exposure during the oocyte stage not only inhibits oocyte maturation, but also promotes injurious effects on *in vitro* fertilization and embryonic development.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), methylglyoxal, and pregnant mare serum gonadotropin (PMSG) were obtained from Sigma (St. Louis, MO). Human chorionic gonadotropin (hCG) was purchased from Serono (NV Organon Oss, The Netherlands). TUNEL *in situ* cell death detection kits were acquired from Roche (Mannheim, Germany), and CMRL-1066 medium from Gibco Life Technologies (Grand Island, NY).

2.2. COC collection and in vitro maturation (IVM)

ICR mice were acquired from the National Laboratory Animal Center (Taiwan, ROC). This research was approved by the Animal Research Ethics Board of Chung Yuan Christian University (Taiwan, ROC). All animals received humane care, as outlined in the Guidelines for Care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, 1984). Mice were maintained on breeder chow (Harlan Teklad chow) with food and water available *ad libitum*. Housing was provided in standard 28 cm × 16 cm × 11 cm (height) polypropylene cages with wire-grid tops, and maintained under a 12 h day/12 h night regimen. Cumulus–oocyte complexes (COCs) were obtained according to a previous protocol (Banwell et al., 2007). Briefly, COCs were isolated from female hybrid ICR mice (21 days old) injected with 5 IU human chorionic gonadotrophin (hCG) 44 h prior to oocyte collection. COCs were collected in HEPES-buffered α-minimum essential medium (MEM) (containing 50 μg/ml Streptomycin sulfate, 75 μg/ml Penicillin G, and 5% fetal bovine serum) by gently puncturing visible antral follicles present on the ovary surface. Germinal vesicle stage oocytes containing an intact vestment of cumulus cells were collected and pooled in at least 8 animals. For oocyte maturation, one drop (~100 μl) of buffer (α-MEM supplemented with 50 μg/ml Streptomycin, 75 μg/ml Penicillin G, 5% FBS and 50 mIU/ml recombinant human FSH) containing 10 COCs was added under oil in 35 mm culture dishes. COC maturation was analyzed following treatment with or without various concentrations of MG (2.5 μM, 5 μM or 10 μM) for 24 h under an atmosphere of 5% O_2, 6% CO_2 and balance of N_2 at 37 °C.

2.3. Maturation status assessment

After *in vitro* maturation (IVM), COCs of each group were treated with 50 U/ml ovine hyaluronidase and gently pipetted for the removal of all cumulus cells. Denuded oocytes were collected, and washed with fresh medium, followed by phosphate-buffered saline (PBS). Oocytes were fixed in ethanol:glacial acetic acid (1:3) for 48 h, and stained with 1% aceto-orcein solution. Nuclear structures were visualized using phase-contrast microscopy.

2.4. In vivo maturation

For obtaining *in vivo* matured oocytes, 21-day-old mice were injected with 5 IU equine chorionic gonadotrophin (eCG) and 5 IU hCG, 61 and 13 h prior to fertilization, respectively. Mature ova were collected from the oviduct into HEPES-buffered α-MEM medium.

2.5. Effects of MG intake on oocyte maturation in an animal model

The effects of MG on oocytes were analyzed in 21-day-old ICR virgin albino mice. Female mice were randomly divided into two groups of 20 animals each, and administered a standard diet with or without 5–20 μM MG in drinking water for 4 days. COCs were collected by pre-treatment with 5 IU human chorionic gonadotrophin (hCG) for 44 h prior to oocyte collection, and analyzed for oocyte maturation, *in vitro* fertilization, and embryonic development.

2.6. In vitro fertilization

For *in vitro* fertilization, ova were washed twice in bicarbonate-buffered α-MEM medium (containing 50 mg/ml Streptomycin, 75 mg/ml Penicillin G and 3 mg/ml fatty acid free bovine serum albumin), and fertilized in the same medium with fresh sperm (obtained from a CBAB6F1 male donor). After incubation with sperm for 4.5 h, eggs were washed three times in potassium simplex optimized medium (KSOM) without amino acids in the presence of L-alanyl-L-glutamine (1.0 mM). Next, eggs were placed in 20 ml drops of KSOM under oil, and cultured overnight. During cleavage to the 2-cell stage, embryos were transferred to a fresh drop of KSOM under oil, and cultured for another 72 h. All fertilization steps and embryo culture were additionally carried out under 5% O_2, 6% CO_2 and balance of N_2 at 37 °C.

2.7. Fertilization assessment

For the examination of fertilization, ova were incubated with sperm for 4.5 h, followed by 3 h of culture in fresh medium. Zygotes were assessed for the presence of the male pronucleus with orcein staining, as described previously (Banwell et al., 2007).

2.8. Cell proliferation

Cell proliferation was analyzed by dual differential staining, which facilitated the counting of cell numbers in inner cell mass (ICM) and trophectoderm (TE) (Chan, 2007; Huang et al., 2007; Pampfer et al., 1990). Blastocysts were incubated with 0.4% pronase in M_2–BSA medium (M_2 medium containing 0.1% bovine serum albumin) for the removal of zona pellucida. Denuded blastocysts were exposed to 1 mM trinitrobenzenesulfonic acid (TNBS) in BSA-free M_2 medium containing 0.1% polyvinylpyrrolidone (PVP) at 4 °C for 30 min, and washed with M_2 (Hardy et al., 1989). Blastocysts were further treated with 30 μg/ml anti-dinitrophenol–BSA complex antibody in M_2–BSA at 37 °C for 30 min, followed by M_2 supplemented with 10% whole guinea pig serum as a source of complement, along with 20 μg/ml bisbenzimide and 10 μg/ml propidium iodide (PI) at 37 °C for 30 min. The immunolysed blastocysts were gently transferred to slides, and protected from light before observation. Under UV light, ICM cells (which take up bisbenzimidine but exclude PI) appeared blue, whereas TE cells (which take up both fluorochromes) appeared orange-red. Since multinucleated cells are not common in preimplantation embryos (Gardner and Davies, 1993), the number of nuclei represent an accurate measurement of cell number.

2.9. TUNEL assay of blastocysts

For TUNEL staining, embryos were washed in MG-free medium, fixed, permeabilized, and subjected to labeling using an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's protocol. Photographic images were obtained with a fluorescence microscope under bright-field illumination.

2.10. Blastocyst development following embryo transfer

To determine the ability of expanded blastocysts to implant and develop *in vivo*, embryos generated were transferred to recipient mice. ICR females (6–8-week-old, white skin) were mated with vasectomized males (C57BL/6J; black skin; National Laboratory Animal Center, Taiwan, ROC) to produce pseudopregnant dams as recipients for embryo transfer. To ensure that all fetuses in pseudopregnant mice were derived from embryo transfer (white color) and not fertilization by C57BL/6J (black color), we examined skin color at day 18 post-coitus. To assess the impact of MG on post-implantation growth *in vivo*, COCs were exposed to 0-10 μM MG for 24 h, followed by fertilization and *in vitro* maturation to the blastocyst stage. Subsequently, 8 untreated control embryos were transferred to the left uterine horn, and 8 MG-treated embryos to the right uterine horn in day 4 pseudopregnant mice. Forty surrogate mice were analyzed and killed on day 18 post-coitus, and the frequency of implantation calculated as the number of implantation sites per number of embryos transferred. The incidence rates of resorbed and surviving fetuses were calculated as number of fetuses per number of implantations, respectively. The weights of the surviving fetuses and placenta were measured immediately after dissection.

2.11. Statistical analysis

Data were analyzed using one-way ANOVA and *t*-tests, and presented as means ± SD. Data were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of MG on oocyte maturation status, fertilization rate, and in vitro embryo development

Although MG evidently induces apoptosis and developmental injury in mouse blastocysts (Hsuuw et al., 2005), the effects of MG on oocyte maturation are currently unclear. Oocyte nuclear maturation status was measured using 10 independent experimental replicates, with ~200 oocytes per group. The number of oocytes