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The regulatory role of icariin on apoptosis in mouse preimplantation embryos with reduced microRNA-21

Chao Zhang, Ya-Ran Shi, Xiao-Ran Liu, Yong-Chun Cao, Jin-Ling Tian, Zi-Ye Jia, Di Zhen, Feng-Hua Liu**, Jian-Ming Gao*

Animal Science and Technology College, Beijing University of Agriculture, Beijing, P.R. China

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ABSTRACT

We constructed a model of apoptosis in mouse preimplantation embryos and investigated the effect of the flavonol icariin on embryonic development *in vitro* in embryos with reduced microRNA-21 (miR-21). The model was generated by microinjecting an miR-21 inhibitor into the cytoplasm of mouse pronuclear embryos, which were cultured *in vitro* using modified CZB (mCZB) basal medium (model group), or using mCZB medium with 0.6 μg/mL icariin as an experimental group (model-Ica). These were compared with embryos collected *in vivo* (*vivo* group) or not microinjected (control group). Developmental rates *in vitro* of two- and four-cell embryos and blastocysts were observed, and Hoechst 33342 and terminal deoxynucleotidyl transferase dUTP nick end labeling staining were used to count blastocyst cell numbers and apoptotic cell numbers and percentages. The transcriptional levels of miR-21, the apoptotic genes *caspase 3* and phosphatase and tensin homolog deleted on chromosome ten (*PTEN*), and the antiapoptotic gene *Bcl-2* were detected by quantitative polymerase chain reaction (qPCR). Western immunoblotting was used to detect the protein levels of *caspase 3*, *PTEN*, and *Bcl-2*. Compared with the model group, icariin treatment significantly improved blastocyst development *in vitro* (58.43 ± 7.53% vs. 37.85 ± 6.47%; $P < 0.01$), whereas it was not significantly different to the control group (60.34 ± 9.86%). Icariin treatment significantly increased the blastocyst cell numbers (47.02 ± 4.93 vs. 37.70 ± 5.80; $P < 0.01$), and reduced the rates of apoptosis (5.51 ± 2.35% vs. 10.11 ± 4.21%; $P < 0.01$), whereas the blastocyst cell numbers and apoptotic rates revealed no significant differences between the *vivo* (46.06 ± 6.50, 5.95 ± 2.56%) and control groups (45.77 ± 4.09, 6.18 ± 2.41%). Icariin treatment significantly improved miR-21 expression in all embryo stages, reduced the transcriptional levels of *caspase 3* and *PTEN*, and increased the levels of *Bcl-2*. The protein expression levels of *caspase 3* and *PTEN* were decreased in blastocysts and the level of *Bcl-2* was increased ($P < 0.01$). These had no significant differences with the *vivo* and control groups, and the protein levels revealed no significant differences between two- and four-cell embryos. Thus, miR-21 was necessary for preimplantation embryonic development, and embryo quality was closely associated with the apoptosis-related protein expression levels regulated by miR-21. Icariin upregulated miR-21 expression and reduced apoptosis in embryos with reduced miR-21. It also improved embryonic developmental quality *in vitro*, indicating an important regulatory role for miR-21 in blastocyst development *in vitro*.

1. Introduction

Preimplantation embryonic production *in vitro* is important in human assisted reproductive technology and animal embryo engineering. In mammals, many embryos show developmental blocking in early development. Some

* Corresponding author. Tel.: +86 10 80799424; fax: +86 10 80791788.
** Alternate corresponding author. Tel./fax: +86 10 80794699.
E-mail addresses: liufenghua1209@126.com (F.-H. Liu), 19836004@bua.edu.cn (J.-M. Gao).

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preimplantation embryos cultured *in vitro* in media with a defined chemical composition often halt at a particular developmental stage instead of finishing development from the zygote to the blastocyst stage; this phenomenon is known as *in vitro* developmental blockage. Some studies have shown that 50% of human embryos arrest in the first week of *in vitro* culture [1], and only 25% of embryos can meet transplant conditions [2]. The bovine blastocyst formation rate *in vitro* is generally about 32% [3] and for the pig it is about 22% [4]. Thus, preimplantation embryo developmental arrest greatly hinders the advance of assisted reproductive technology and livestock production. Although such arrest is very important, the mechanism is still not clear. At present, the reasons for preimplantation embryonic development arrest are complex and might be related to genetic or environmental factors. Apoptosis is one of the main factors causing embryonic developmental arrest [5].

Apoptosis is an autonomous program of cell death controlled by genes and participates in a great number of biological reactions in the body. It is triggered by the activation, expression, and regulation of a series of apoptotic gene families such as the Bcl family [6] and caspase family [7], as well as by microRNAs (miRNAs) [8,9] and signaling pathways such as p53 [10] and nuclear factor κB [11]. MicroRNAs are endogenous small RNA molecules (20–24 nt) that are widely found in animals and plants, and have an important regulatory role in cell proliferation, apoptosis, and differentiation through degrading mRNA or hindering the translation of target genes [12,13]. MicroRNA-21 (miR-21) frequently plays an antiapoptotic role in many cellular processes and functions [14,15]. The antiapoptotic ability of miR-21 has been linked to diabetes [16], heart disease [17], cancer [18], and many other diseases. It also plays an important role in embryo implantation, placentation, and abortion [19,20]. In addition, there is a close link between miR-21 and preimplantation embryo development. Adding recombinant interleukin 6 to the culture medium activated the interleukin 6 and Stat3 pathway, significantly increased miR-21 expression, and improved the viability of mouse preimplantation embryonic development *in vitro* by increasing blastocyst cell numbers and preventing apoptosis [21]. In our previous study, we also found that a flavonol from Chinese medicine, icariin, could significantly enhance the expression of miR-21 in mouse blastocysts and played an antiapoptotic role in promoting embryonic development [22]. The expression of miR-21 affects preimplantation embryonic development and arrest *in vitro*, but the regulatory role and pathway of miR-21 have not been defined clearly.

Icariin is extracted from *Epimedium* stems and leaves and is a kind of prenylflavonoid compound [23,24]. Modern pharmacology studies have shown that icariin can take part in a variety of important biological activities and has immunomodulatory, antitumor, and endocrine functions and plays a role in protecting the cardiovascular system. Icariin is used in traditional Chinese medicine and has broad functions in pharmacology and biology [25]. In recent years, icariin has been found to function as an antiapoptotic agent [26]. Icariin also has antioxidant effects and can protect against free radical–mediated oxidative damage [27]. Additionally, icariin has a significant role in promoting cell differentiation, such as osteoblast differentiation and bone formation [28], and can induce embryonic stem cells to differentiate into cardiomyocytes [29,30]. However, the effect of icariin on preimplantation embryonic development has rarely been studied. Our previous studies have shown that icariin can promote the blastocyst development rate of parthenogenetically activated oocytes and *in vitro* fertilization in the pig. It can reduce the levels of nitric oxide in the culture environment, inhibit the production of malondialdehyde, and reduce free radical damage to different stages of development in embryos [31–33]. Icariin, through the activation of miR-21, exerts antiapoptotic effects, and enhances the development rates and quality of blastocyst formation from mouse pronuclear embryos cultured *in vitro*, proving that miR-21 is one target of icariin [22]. However, previous studies did not validate the regulatory role and mechanisms of miR-21 in preimplantation embryo development and did not investigate the regulatory effect of icariin in embryos with reduced miR-21. In this study, we generated apoptosis-prone mouse preimplantation embryos with reduced miR-21 by cytoplasmic microinjection of an miR-21 inhibitor into pronuclear embryos, and explored the regulatory effect of icariin on apoptosis *in vitro*.

2. Materials and methods

2.1. Reagents and animals

All reagents used were from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Specific pathogen-free Kunming white mice were from the Chinese Academy of Military Sciences, and our experiments complied with the regulations of the Chinese Academy of Sciences for experimental animal breeding and use. Mice had free access to water and food, and were subjected to a light-dark cycle of 14 hours of light and 10 hours of dark.

2.2. Collection of embryos in vivo

Female mice were superovulated by intraperitoneal injection with 10 IU of pregnant mare's serum gonadotropin (Ningbo second hormone factory, Ningbo, China) and 10 IU of human chorionic gonadotropin (Ningbo second hormone factory) at 48-hour intervals, and caged with fertile male mice for mating. After 47 and 56 hours, respectively, two- and four-cell embryos were obtained from the oviducts. Blastocysts were collected from uteri at 96 hours. All embryos were rinsed with PBS (Gibco) and stored at −80 °C. This group of embryos was used as the *in vivo* group (*vivo* group).

Collection of pronuclear embryos: superovulation of mice was conducted as described previously. At 25 to 26 hours after human chorionic gonadotropin injection, pronuclear embryos were released from the excised oviducts. Cumulus cells were removed using 0.1% hyaluronidase and the embryos were washed three times with PBS for subsequent culture *in vitro*.

2.3. Construction and verification of a reduced miR-21 mouse preimplantation embryo model

Mouse pronuclear embryos were selected randomly and placed in 50 μL culture droplets of M_2 medium covered