Examination of the Role of p53 in Embryo and Sperm Function

Nida Gunay
B.Med.Sc.

Department of Physiology
Faculty of Medicine
The University of Sydney

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Supervisor: A/Professor Chris O'Neill
Human Reproduction Unit, Dept. of Physiology, University of Sydney, Royal North Shore Hospital, St Leonards, 2065.

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ABSTRACT

Assisted reproductive technologies (ARTs) are very efficient in producing embryos, however many of these embryos have poor viability. No more than 50% of IVF embryos complete preimplantation development (Hardy et al. 2001). The poor viability is manifested as a reduced rate of cell proliferation and increased rates of apoptosis in the early embryo, resulting in high rates of embryo mortality (Hardy et al. 2001). The reduced viability occurs as a response to a range of cellular stressors that are a consequence of embryo culture (Hardy et al. 2001). The stress of culture disrupts some survival signalling pathways, metabolism of substrates and induces redox stress (Hardy et al. 2001).

The cellular stress sensor p53 is expressed in the early embryo but is normally kept at very low levels (Li et al. 2005). This latency may be breached in IVF embryos following culture of zygotes *in vitro* for 96 hours, resulting in the up-regulation and nuclear accumulation of p53 (Li et al. 2005). Activation of the p53 stress-sensing pathway in the early mouse embryo by culture *in vitro* causes a marked loss of their developmental competence (Li et al. 2005).

This study aimed to establish whether benefits could be obtained by culturing mice IVF embryos in the presence of p53 protein inhibitors. IVF zygotes were cultured individually in 10µl drops of 1.25, 2.5, 5 or 10µM Pifithrin-α (PFTα) in 0.05% DMSO for 96 hours. On day 5 the development stage was assessed. Embryos reaching the blastocyst stage

were fixed and stained with Hoechst 33342 for total cell count and the proportion of nuclei with normal and abnormal morphology. There was an increase in the blastocyst rate, total cell count and the proportion of nuclei in a blastocyst with normal nuclei in 10µM-treated embryos.

This study also aimed to determine whether benefits could be obtained by incubating mouse IVF sperm with p53 protein inhibitors during IVF. IVF sperm was treated with 1.25, 2.5, 5 or 10µM of PFTα in 0.05% DMSO during incubation with oocytes for 6 hours. Resulting zygotes were cultured for 96 hours individually in 10µl drops of MOD-HTFM. On day 5 the development stage was assessed. Embryos reaching the blastocyst stage were fixed and stained with Hoechst 33342 for total cell count and the proportion of nuclei with normal and abnormal morphology. There was a reduction in the proportion of fragmented nuclei in blastocysts derived from 1.25 and 10µM-treated sperm. 10µM-treated sperm increased the total cell count, the proportion of normal nuclei in a blastocyst and the blastocyst development rate. IVF sperm incubated with 1.25µM PFTα during insemination of oocytes increased the fertilisation rate.

Another aim of this study was to establish whether p53 siRNA could inhibit p53 mRNA in mice IVF embryos and if so, whether this would improve embryo viability in culture. IVF zygotes were transfected with 15nM p53 small inhibiting RNA (siRNA) and 0.8% Oligofectamine Reagent immediately, 24 h, 48 h and 72 h after IVF then cultured individually in 10µl drops of MOD-HTFM for a total of 96 hours. On day 5 the blastocyst rate was assessed and immunofluorescence performed probing for p53. There

was no significant reduction in p53 expression and no improvement in blastocyst rate at any of the transfection times. However, there was a decrease in the proportion of nuclei which expressed p53 when p53 siRNA was transfected 72 hours after IVF. Also, it was determined that siRNA was efficiently being delivered into the preimplantation embryo with Oligofectamine Reagent.

Lastly, this study aimed to determine whether mice sperm with p53 gene deletions have a selective advantage in fertilising the oocyte compared to their wild-type counterparts. p53+/- males were mated with p53+/+ females and the resulting zygotes genotyped after 24 hours of culture. More than 50% of offspring had a p53+/+ genotype. There was no selective advantage for p53 null sperm to fertilise the oocyte, there was actually a disadvantage.

The selective disadvantage for p53 null sperm to fertilise the F1 hybrid oocyte in IVF compared to its wild-type counterparts may imply that p53 null sperm are not as viable and may have a survival disadvantage. The reduction in fertility of p53 null sperm *in vitro* infers that p53 function may be important for the fertility of the mouse sperm *in vitro*.

The results of this thesis could establish means of improving human embryo viability in ART, some examples being P53 protein inhibition in preimplantation embryos during culture prior to transfer to the uterus, or P53 protein inhibition in IVF sperm. The use of the new technology, p53 siRNA was not effective in inhibiting p53 expression, although

the build-up experiments determined that siRNA is efficiently delivered into the preimplantation embryo with Oligofectamine Reagent. The demonstration that p53 null sperm has a selective disadvantage in fertilising the oocyte compared to their wild-type counterparts does not indicate a positive selection pressure for naturally occurring mutations to this gene. And so, there is no concern regarding the genetic and epigenetic risks to progeny arising from assisted reproductive technologies with respect to sperm.

DECLARATION

I declare that this thesis is my own account of my own research and contains, as its main content, work which has not been submitted for a degree at any University. For the studies involving the analysis of p53 protein expression, the experiments were performed by me but the western analysis was performed by Omar Chami.

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Apart from standard abbreviations for concentration, volume and mass, the following abbreviations were used in this thesis:

ART	Assisted reproductive technology
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
Dox	Doxorubicin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ES	Embryonic stem
FBS	Fetal bovine serum
FCS	Fetal calf serum
Flush-HTFM	Flush-human tubal fluid medium
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
H_2O_2	Hydrogen peroxide

HBSS	Hank's balanced saline solution
hCG	Human chorionic gonadotrophin
HRP	Horse Raddish Peroxidase
HRU	Human Reproduction Unit
Hsp	Heat shock protein 70.1
[^{3}H]TdR	^{3}H-labeled thymidine
HTF	Human tubal fluid medium
ICM	Inner cell mass
ICSI	Intra cytoplasmic sperm injection
IGF	Insulin-like growth factor
i.p.	Intra peritoneal
ISF	*In situ* fertilisation
I.U.	International Units
IVF	*In vitro* fertilisation
LH	Luteinising hormone
MOD-HTFM	Modified human tubal fluid medium
mRNA	Messenger ribonucleic acid
OCS	Oestrous cow serum
PAF	Platelet-activating factor
Pafah1b a_1	PAF:acetylhydrolase 1b a_1 subunit
PAFR	Platelet-activating factor receptor
PCR	Polymerase chain reaction
PFTα	Pifithrin-α

PI3K	Phosphatidylinositol 3-kinase
PMSG	Pregnant mares serum gonadotrophin
PVA	Polyvinyl alcohol
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
s.d.	Standard deviation
siRNA	Small interfering RNA
SOF	Synthetic oviduct fluid
TAE	Tris-acetate-EDTA
TNF	Tumour necrosis factor
topo	Topoisomerases