

Examination of the Role of p53 in Embryo and Sperm Function

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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 10 μ M-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 10 μ M of PFT α in 0.05% DMSO during incubation with oocytes for 6 hours. Resulting zygotes were cultured for 96 hours individually in 10 μ l 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 10 μ M-treated sperm. 10 μ M-treated sperm increased the total cell count, the proportion of normal nuclei in a blastocyst and the blastocyst development rate. IVF sperm incubated with 1.25 μ M PFT α 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 10 μ l 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.

Nida Gunay

TABLE OF CONTENTS

		Page Number
	TITLE PAGE	I
	ACKNOWLEDGMENTS	II
	ABSTRACT	III
	DECLARATION	VII
	TABLE OF CONTENTS	VIII
	LIST OF TABLES	XIV
	LIST OF FIGURES	XVII
	LIST OF ABBREVIATIONS	XXI
Chapter 1	LITERATURE REVIEW	1
1.1	Introduction	2
1.2	<i>In Vitro</i> Fertilisation	4
1.2.1	History of IVF	4
1.2.2	IVF Today	5
1.3	IVF and Cellular Stress	6
1.3.1	Growth and Survival Factor Deprivation	6
1.3.2	Metabolic and Substrate Imbalance	13
1.3.3	Redox Stress	15
1.3.4	Accumulation of Toxins	18
1.3.5	Gross or Minor Chromosome Aberrations	20

1.3.6	Media Contaminants	23
1.3.7	Conclusion	25
1.4	p53 Activation by the Stressors of Embryo Culture	25
1.4.1	Introduction	25
1.4.2	Metabolic and Substrate Imbalance	26
1.4.3	Reactive Oxygen Species (ROS)	27
1.4.4	Conclusion	30
1.5	P53	31
1.5.1	Biology of p53	31
1.5.2	p53 Activation	32
1.5.2.1	DNA Damage	33
1.5.2.2	Aberrant Growth Survival Signals	35
1.5.2.3	Chemotherapeutic Drugs, Ultra-Violet Light, Protein-Kinase Inhibitors	37
1.5.3	Downstream Genes of p53	40
1.5.3.1	Cell-Cycle Inhibition	40
1.5.3.2	Apoptosis	41
1.5.3.3	Genetic Stability	42
1.6	p53 Inhibitors	42
1.6.1	Pifithrin- α (PFT α)	42
1.6.2	Small Interfering RNA (siRNA)	44
1.6.3	Conclusion	46
1.7	p53 and Embryos	46

1.8	p53 and Sperm	49
1.9	Aims of the Thesis	50
Chapter 2	GENERAL MATERIALS AND METHODS	54
2.1	Animal Husbandry	55
	2.1.1 Animals	55
	2.1.2 Superovulation of Female Mice	55
	2.1.3 Mating of Mice	56
2.2	Media Preparation	56
	2.2.1 Collection Media	56
	2.2.2 Culture Media	57
2.3	Chemicals	58
	2.3.1 Pronase	58
	2.3.2 Pifithrin- α (PFT α)	58
	2.3.3 Hyaluronidase	59
	2.3.4 Hoechst 33342	59
	2.3.5 Formaldehyde	59
2.4	Embryo Manipulations	60
	2.4.1 Recovery of Zygotes	60
	2.4.2 Recovery of Blastocysts	62
	2.4.3 Embryo Culture	63
	2.4.4 Blastocyst Staining and Cell Count	63
2.5	Polymerase Chain Reaction (PCR)	64
	2.5.1 Zona Pellucida Removal	64

2.5.2	DNA Extraction of 2-cell Embryos	65
2.5.3	Master Mix	65
2.5.4	PCR	66
2.5.5	Agarose Gel	66
2.6	Small Interfering RNA (siRNA)	67
2.6.1	Media Preparation	67
2.6.2	siRNA-Oligofectamine Complex Preparation	67
2.6.3	Incubation with siRNA	68
2.7	Immunofluorescence	69
2.7.1	Fixing and Blocking Non-Specific Binding	69
2.7.2	Incubation with Primary Antibody	69
2.7.3	Incubation with Secondary Antibody and Analysis	70
2.8	<i>In Vitro</i> Fertilisation	71
2.8.1	Superovulation of female mice	71
2.8.2	Sperm Collection	71
2.8.3	Recovery of Oocytes	72
2.8.4	Sperm Count	73
2.8.5	Embryo Culture	73
2.9	Western Blot Analysis	74
2.9.1	Extracting proteins from IVF media	74
2.9.2	Size separation of proteins	75
2.9.3	Blotting the Proteins	75
2.9.4	Incubation with Antibodies	76

2.9.5	Chemiluminescent detection and film exposure	76
2.10	Analysing Data	77
2.10.1	Graphing	77
2.10.2	Statistical Analysis	78
Chapter 3	EFFECT OF PFTα ON IVF EMBRYOS AND SPERM	80
3.1	Introduction	81
3.2	Materials and Methods	83
3.2.1	Experimental Procedures	83
3.3	Results	85
3.4	Discussion	96
Chapter 4	EFFECT OF p53 siRNA ON EMBRYO DEVELOPMENT AND p53 EXPRESSION	98
4.1	Introduction	99
4.2	Materials and Methods	100
4.2.1	Experimental Procedures	100
4.3	Results	104
4.4	Discussion	122
Chapter 5	EFFECT OF p53 NULL SPERM ON FERTILISATION	124
5.1	Introduction	125
5.2	Materials and Methods	125
5.2.1	Experimental Procedures	125
5.2.2	Statistical Analysis	127
5.3	Results	127

5.4	Discussion	132
Chapter 6	GENERAL CONCLUSIONS	133
6.1	Treatment of IVF Embryos with Pifithrin- α	134
6.2	Treatment of IVF Sperm with Pifithrin- α	136
6.3	Pifithrin- α	139
6.4	Treatment of IVF Embryos with p53 siRNA	143
6.5	Fertilising Capacity of Sperm with p53 Gene Deletions	144
6.6	Conclusion	146
Chapter 7	REFERENCES	149
	APPENDICIES	156
	Appendix I	157
	Appendix II	159
	Appendix III	162
	Appendix IV	167
	Appendix V	169
	Appendix VI	171

LIST OF TABLES

	Page Number
Table 1. Effect of PFT α on the proportion of IVF embryos that developed to blastocysts by 96 and 120 hours	86
Table 2. Effect of PFT α -treated sperm on the fertilisation rate of IVF oocytes	94
Table 3. Effect of PFT α -treated sperm on the proportion of blastocysts in IVF embryos by 120 hours	94
Table 4. Effect of SUPERase.In and RNasequre Reagent on the degeneration of embryos 72 hours after zygote collection	104
Table 5. Effect of 3, 6 and 12nM Signal Silence Control siRNA on the degree of fluorescein staining in day 1 to 5 embryos	109
Table 6. Effect of Oligofectamine Reagent on the degree of fluorescein staining in day 2, 3 and 4 embryos	111
Table 7. Effect of p53 siRNA transfection with Oligofectamine Reagent immediately after IVF on blastocyst rate and total cell count	112

Table 8. Effect of p53 siRNA transfection with Oligofectamine Reagent 72 hours after IVF on blastocyst rate	113
Table 9. Effect of p53 siRNA transfection with Oligofectamine Reagent 72 hours after IVF on the number of cells in an embryo which stained brighter for p53 than their cytoplasm	113
Table 10. Effect of p53 siRNA transfection with Oligofectamine Reagent 24 hours after IVF on blastocyst rate	116
Table 11. Effect of p53 siRNA transfection with Oligofectamine Reagent 24 hours after IVF on the number of cells in an embryo which stained brighter for p53 than their cytoplasm	116
Table 12. Effect of p53 siRNA transfection with Oligofectamine Reagent immediately after IVF on the number of cells in an embryo which stained brighter for p53 than their cytoplasm	118
Table 13. Effect of p53 siRNA transfection with Oligofectamine Reagent immediately after IVF on the number of nuclei in an embryo that had nucleolar staining	118

Table 14. Effect of p53 siRNA transfection with Oligofectamine

Reagent 48 hours after IVF on blastocyst rate

121

Table 15. Effect of p53 siRNA transfection with Oligofectamine

Reagent 48 hours after IVF on the number of cells in an embryo

which stained brighter for p53 than their cytoplasm

121

LIST OF FIGURES

Figure 1. An illustration of the structure of the P53 protein	32
Figure 2. Post-translational modification and activation of P53 in response to DNA damage	35
Figure 3. Illustration of the stimuli that activate P53 and their post-translational modifications and protein-protein interactions	39
Figure 4. Typical IVF blastocysts 96 hours after zygote culture which were stained with Hoechst 33342	87
Figure 5. The effect of PFT α on the number of abnormal, normal and total cells in IVF blastocysts	89
Figure 6. The expression of p53 in IVF media via Westerns at 0, 1, 2, 3 and 4 hours after insemination	90
Figure 7. The effect of PFT α -treated sperm on the fertilisation rate in IVF when 50, 100, or 200X10 ³ sperm/ml were used in insemination	91

- Figure 8.** The effect of PFT α -treated sperm on the blastocyst rate in IVF when 50, 100, or 200X10³ sperm/ml were used in insemination 91
- Figure 9.** The effect of PFT α -treated sperm on the number of abnormal, normal and total cells in IVF blastocysts 93
- Figure 10.** The effect of PFT α -treated sperm on the number of abnormal nuclei in IVF blastocysts 95
- Figure 11.** The effect of 0.1 μ m filters and nuclease free BSA in culture media on total cell counts in blastocysts 105
- Figure 12.** The effect of Oligofectamine Reagent on the number of total cells in blastocysts 106
- Figure 13.** The intensity of FITC labelling in day 2, 3, 4 and 5 embryos cultured in 50nM Signal Silence Control siRNA (Fluorescein Conjugate) 107
- Figure 14.** The intensity of FITC labelling in day 2 embryos cultured in 12.5, 25 and 50nM Signal Silence Control siRNA (Fluorescein Conjugate) 108

Figure 15. Day 2 embryos labelled with Signal Silence Control siRNA (Fluorescein Conjugate) with and without Oligofectamine Reagent	111
Figure 16. Typical blastocysts transfected with p53 siRNA 72 hours after IVF and probed for p53 by immunofluorescence after 96 hours of culture	115
Figure 17. Typical blastocysts transfected with p53 siRNA 24 hours after IVF and probed for p53 by immunofluorescence after 96 hours of culture	117
Figure 18. Cytoplasmic p53 expression of blastocysts transfected with p53 siRNA immediately after IVF then cultured for 96 hours	119
Figure 19. Typical blastocysts transfected with p53 siRNA immediately after IVF and probed for p53 by immunofluorescence after 96 hours of culture	120
Figure 20. The effect of p53 null sperm on the proportion of genotypes in progeny after IVF and ISF between p53 ^{+/-} males and F1 (+/+) females	131

Figure 21. The effect of p53 null sperm on the proportion of genotypes in progeny after IVF between p53^{+/-} males and C57BL (+/+) females

131

Figure 22. The effect of PFT α -treated sperm on the fertilisation rate when 50X10³ sperm/ml was used in the insemination in six replicates

137

LIST OF ABBREVIATIONS

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 ₂ O 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	<i>In situ</i> fertilisation
I.U.	International Units
IVF	<i>In vitro</i> 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
PFT α	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