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Original article

Effects of paternal heat stress on the in vivo development of preimplantation embryos in the mouse

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Abstract – The objective of this study was to examine the effect of paternal heat stress on the in vivo development of preimplantation embryos in the mouse. Synchronised B6CBF1 female mice were mated either to a control male mouse or to one that had been exposed at 7, 21 or 35 days previously, for 24 h to an ambient temperature of 36 ± 0.3 °C and 66 ± 5.6% relative humidity. Embryos were collected from the oviducts of mice at 14–16 h, 34–39 h or 61–65 h after mating or from the uterus at 85–90 h after mating and their developmental status was evaluated morphologically. The number of cells within blastocysts was also determined using bisbenzimide-propidium iodide staining. Paternal heat stress 7 days before mating reduced the proportion of embryos developing from 4-cell (4-C) to morulae (M), hatched blastocysts, total blastocysts and the number of inner cell mass (ICM) and trophectoderm (TE) cells in the blastocyst. Paternal heat stress 21 days prior to mating reduced the proportion of 2-C and 4-C to M embryos with no embryos developing to blastocysts. There were also increases in the number of 1-C and abnormal embryos recorded at this time. Paternal heat stress 35 days before mating decreased the proportion of 2-C embryos, expanded blastocysts and ICM and TE cells in the blastocyst. These results support previous work demonstrating that both the sperm in the epididymis and germ cells in the testis are susceptible to damage by environmental heat stress, with spermatocytes being the most vulnerable. This study also demonstrates that subtle effects on the male such as a short exposure to elevated environmental temperatures can translate to quite profound paternal impacts on early embryo development.

paternal heat stress / in vivo development / mouse preimplantation embryos

1. INTRODUCTION

In most mammals, testicular temperature is maintained between 2 and 7 °C lower than the core body temperature, and slightly higher than the scrotal temperature [1–4] for normal spermatogenesis. Any factors causing an increase in testicular temperature (e.g. high ambient temperature, local heat stress) can damage spermatogenesis, which may result in decreased sperm numbers and reduced sperm motility in the ram [5] and boar [6], normal fertilisation, but an increase in embryonic death in the pig [6], mouse [7–9], rabbit [10, 11], sheep [12–17] and rat [18].

The quality of semen in man has also been reported to vary with the season and

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occupation. Sperm concentration, total sperm count per ejaculate and motile sperm concentration have been reported to be lower in the summer than in the winter [19–22] and even mild heat can temporarily reduce the number of sperm ejaculated after two to three weeks [23]. The effect of heat stress on spermatogenesis may further extend time to pregnancy and decrease birth rate in women [24].

Early studies have shown that paternal heat stress reduces fertilisation rates [8] and the in vivo development of preimplantation embryos in the mouse [7]. The extent of the effect could be related to the time after heat-stress of the males [8, 18]. Setchell et al. [18] reported that local heating of the testis between 7 days and 35 days prior to mating reduces the size and weight of litters in the mouse. Our own studies have also indicated that the in vitro developmental rates of heat-sired preimplantation embryos begin to decrease in the embryos sired on 3 days after heat-stress, were the lowest when embryos were sired on 21 days after heat-stress, and then gradually returned to control levels when sired on 42 days [9]. Therefore, in the present study, we investigated the effect of paternal heat stress on the in vivo development of preimplantation embryos conceived at 7, 21 and 35 days after heating of the male to determine the stage at which the preimplantation embryos might be affected by paternal heat stress.

2. MATERIALS AND METHODS

Procedures used for all experimental animals were approved by the Animal Ethics Committee, at the University of Adelaide.

2.1. Heat stress of the male

Twelve fertile B6CBF1 male mice (3 to 4 months old) were placed in a psychrometric chamber set at 36 ± 0.3 °C and 65.8 ± 5.6% relative humidity and heated for 24 h, while another twelve comparable fertile F1 male mice were similarly kept at 21 °C as a control group. This experiment was undertaken in triplicate.

2.2. Determination of sperm characteristics

The control and heat-stressed males were killed by cervical dislocation, 7, 21 and 35 days after the treatment. The epididymides were collected and the cauda epididymis was isolated and placed in a Falcon tube containing 2 mL of HTF medium (pH 7.4). The tissue of the cauda epididymidis was minced by using scissors to release spermatozoa. The spermatozoa were allowed to swim up and incubated for 1 h in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37 °C, the concentration and motility of spermatozoa were determined, using Microcell chambers (Conception Technologies, USA). For the determination of sperm concentration, 6 μL of the diluted semen were loaded into a Microcell chamber. An Olympus phase-contrast microscope fitted with an eyepiece reticle (Olympus Australia Pty. Ltd. Australia) was used to score sperm from 6 or 12 fields in each side of the chamber. Two hundred to three hundred spermatozoa were scored in each chamber. For the determination of motile sperm, semen was loaded into a prewarmed (37 °C) microcell chamber and placed on the heated (37 °C) microscope stage. The non motile and total motile (any type of movement) sperm were counted by looking through the reticle grid in the microscope eyepiece. Two hundred spermatozoa were scored in each Microcell chamber. The viability and morphology of spermatozoa were evaluated with a staining method of eosin-nigrosin [25]. Ten microliters of diluted semen stained with a solution containing 0.67% eosin (Sigma, Australia) and 5% nigrosin (Sigma, Australia) was applied to a slide which was kept on a warm plate at 37 °C and smeared using a coverslip (18 × 18 mm). The slides were left to dry on the warm plate for about 5 min. The percentage live (unstained, white in colour)/dead (stained, pinkish in colour) spermatozoa was determined by counting 200 sperm

cells in each of three prepared slides using an Olympus bright field microscope fitted with eyepiece reticles (Australia) (400×). The prepared slides were also used for morphological evaluation. Normal sperm showed no sign of defect but had the ideal sperm cell structure. Abnormal sperm showed defects with the head, midpiece or tail.

2.3. Oestrous synchronisation of female mice

Normal B6CBF1 female mice (2 to 3 months old) were placed in a room with no males present. After 28 days, a small cage containing male mice was placed on top of a large cage containing female mice so that the "male effect" would shorten and synchronise the female oestrous cycles [26, 27]. Approximately 30% of female mice were in oestrus on the third day following exposure to the male, and showed vaginal gaping, with vaginal tissues being lighter pink and less moist, and the striations being more pronounced.

2.4. Mating and collection of preimplantation mouse embryos

Oestrous female mice were mated with control or heat-stressed males. Each control male and heat-stressed male was caged with 2 oestrous females on 7, 21 and 35 days after heating of the male. The existence of a copulation plug the following morning, was taken as an indication of a successful mating.

Successfully mated mice were euthanised by cervical dislocation at specific intervals after mating. The mating time was defined as midnight (0.00 h) and female mice were allocated randomly for the collection of preimplantation embryos at the zygote, 2-cell, 4-cell to morula and blastocyst stages from the oviduct or the uterus at 14–16 h, 34–39 h, 61–65 h and 85–90 h after mating, respectively. The number of corpora lutea, the number of preimplantation embryos recovered and their stages of development were recorded for each female.

2.5. Morphological evaluation of preimplantation embryos

The quality of the individual embryos was determined by a method described previously [28], scoring normal, abnormal or unfertilised. The definitions applied were:

2.5.1. Normal

The embryo was spherical and symmetrical with cells of uniform size, colour and texture; the embryo was at an appropriate stage of development, i.e. the embryos recovered at 14–16 h, 34–39 h, 61–65 h and 85–90 h after mating were zygotes judged by the presence of the second polar body or pronuclei, 2-cell embryos, 4-cell embryos to morulae and morulae to blastocysts, respectively.

2.5.2. Abnormal

The embryos showed extruded blastomeres, a few cells of variable size, and contained numerous large vesicles.

2.5.3. Unfertilised

An ovulated ovum which did not contain the second polar body or pronuclei.

2.6. Differential fluorescent labelling of trophectoderm (TE) and inner cell mass (ICM) in the blastocyst

The number of TE and ICM cells was determined by a method described previously [28]. Blastocysts were transferred from a Hepes-HTF medium [29] to acid Tyrode solution [30], under constant observation for 30–60 s, until the zonae pellucidae were completely dissolved. They were then washed 3 times in the Hepes-HTF medium and transferred into a CZB medium [31] containing 10% rabbit anti-mouse whole serum (M5774, Sigma, Australia), and then incubated at 37 °C for 15 min. The embryos were washed 3 times in the Hepes-HTF medium and transferred to the CZB medium containing 10% guinea pig serum complement (51639, Sigma, Australia) and incubated at 37 °C for 5–6 min. They were then

Table I. Semen characteristics of male mice, 7, 21 and 35 days after the heated treatment.

Groups*	C ($N = 18$)	H7 ($N = 6$)	H21 ($N = 6$)	H35 ($N = 6$)
Sperm concentration ($\times 10^6 \cdot mL^{-1}$)	13.5 ± 0.37^a	9.1 ± 0.58^b	6.5 ± 0.76^c	10.2 ± 0.37^b
Sperm motility (%)	68.5 ± 2.13^a	44.2 ± 5.23^b	30.5 ± 2.89^c	54.2 ± 3.74^b
Sperm viability (%)	69.6 ± 1.12^a	62.5 ± 2.05^b	36.7 ± 1.63^c	66.7 ± 1.26^a
Sperm abnormality (%)	6.5 ± 0.46^a	18.1 ± 1.39^b	38.3 ± 1.06^c	12.5 ± 0.76^d

* At each time point, 6 controlled and 6 heated males were killed for the determination of semen characteristics. The data obtained from 18 controlled males (C) were pooled together because there were no significant differences in sperm concentration, motility, viability and abnormality of controlled males between day 7, day 21 and day 35.
The values given are mean ± SEM. The values with different alphabetical superscripts differ significantly ($P < 0.01$–0.001) in semen characteristics between controls and heated groups and between heated groups in days 7 (H7), 21 (H21) and 35 (H35) after the treatment (ANOVA analysis).

washed 3 times in the Hepes-HTF and stained in the CZB medium containing 20 $\mu g \cdot mL^{-1}$ bisbenzimide (Hoechst dye) (B2883, Sigma, Australia) with 10 $\mu g \cdot mL^{-1}$ propidium iodide (PI) (P4170, Sigma, Australia) at 37 °C. After 30–40 min, the embryos were fixed in 1% paraformaldehyde in PBS for 1 min and washed 3 times in the Hepes-HTF. The embryos were then transferred to a slide in a droplet of less than 5 µL, covered with a cover glass and squashed gently with the blunt end of a pen. The area under the cover glass was filled with the Hepes-HTF and nail varnish (Supershine, North Ryde, Australia) was used to seal around the cover glass. Finally, the differential labelled nuclei were examined using an Olympus fluorescent microscope (Japan) within a range of 330 to 385 nm of UV exciting light and of 410 to 600 nm of emission in blue. The total magnification in the eyepiece and photograph was ×400 and ×250, respectively. PI-labelled nuclei (dead cells /TE cells) appeared red while the Hoechst B2883 labelled nuclei (viable/ICM cells) appeared blue.

2.7. Data analysis

Comparisons of differences between groups were made using analysis of variance. A significant difference was assumed to exist when the probability of it being due to chance was less than 5%.

3. RESULTS

3.1. Semen characteristics of male mice, 7, 21 and 35 days after heat stress

Heat stress significantly reduced ($P < 0.01$–0.001) sperm concentration, motility, and viability of male mice at 7, 21 and 35 days when the greatest decrease occurred at 21 days after the treatment (Tab. I). However, the percentage of abnormal spermatozoa increased significantly ($P < 0.01$–0.001) for mice at 7, 21 and 35 days with the greatest increase observed at 21 days after the treatment (Tab. I).

3.2. The effect of paternal heat stress 7 days prior to mating on the in vivo development of mouse embryos

Paternal heat stress 7 days prior to mating did not affect the proportion of embryos developing to the zygote and 2-cell stages at 14–16 h and 34–39 h after mating, but significantly decreased the percentage of 4-cell to morulae at 61–65 h after mating and hatched blastocysts at 85–90 h after mating (Tab. II). However, the number of zygotes and morulae were higher in the heat-sired group (H7) (Figs. 1B and 1E) than in the controls (C7) (Figs. 1A and 1D) when the embryos were collected at 61–65 h and 85–90 h respectively after mating (Tab. II).

Table II. The effect of paternal heat stress 7 days prior to mating on the in vivo development of embryos.

Time of embryos recovered after mating (h)	14–16		34–39		61–65		85–90	
Groups	C7	H7	C7	H7	C7	H7	C7	H7
No. females	4	4	4	4	4	4	7	11
No. corpora lutea	10.8 ± 1.71	10.5 ± 1.29	8.8 ± 1.26	9.3 ± 2.22	7.8 ± 1.89	8.0 ± 0.82	8.3 ± 1.60	8.3 ± 1.56
Recovered embryos	10.3 ± 1.89	10.5 ± 1.29	8.5 ± 1.29	8.3 ± 1.50	7.8 ± 1.89	7.8 ± 0.96	7.7 ± 1.70	7.5 ± 1.64
Zygotes	9.5 ± 0.58	10.5 ± 1.29	0.5 ± 1.00	0.8 ± 0.50	0.3 ± 0.5	2.5 ± 0.82**		
2-C[a]			8.0 ± 2.16	7.3 ± 1.50	0.0 ± 0.00	0.5 ± 0.58		
4-C to M					7.5 ± 2.38	4.0 ± 2.45*	0.0 ± 0.00	1.3 ± 1.42**
B							4.9 ± 2.61	6.2 ± 2.23
HB							2.9 ± 3.20	0.0 ± 0.00***
B + HB							7.7 ± 1.60	6.2 ± 2.23*
Abnormal	0.8 ± 1.50	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.50	0.0 ± 0.00	0.8 ± 0.50	0.0 ± 0.00	0.0 ± 0.00
Unfertilised					0.0 ± 0.00	0.3 ± 0.50		

[a] C: cell, M: morula, B: blastocyst, HB: hatched blastocyst, Abnormal: abnormal embryo, Unfertilised: unfertilised ovum.
The values given are mean ± SEM. The values with *, ** and *** superscripts differ significantly ($P < 0.05$–0.001) between the heat-sired group (H7) and controls (C7) for embryo development (ANOVA analysis).

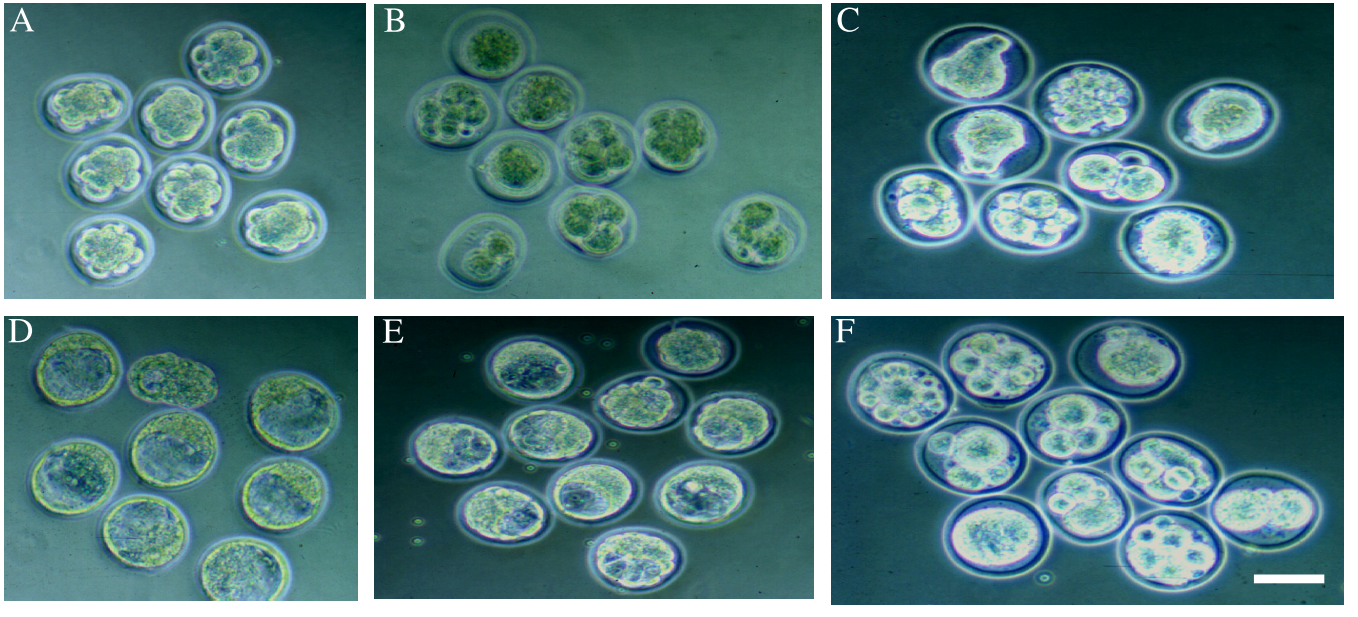

Figure 1. A, B, C represent respectively embryos collected from the oviducts of mice at 64 h after mating with a control male (A) or one 7 (B) or 21 days (C) after heating; D, E and F represent embryos collected from the uterus of mice at 89 h after mating with a control male (D) or one 7 (E) and 21 days (F) after heating ×100 (Bar = 100 μm).

3.3. The effect of paternal heat stress 21 days prior to mating on the in vivo development of mouse embryos

Paternal heat stress 21 days earlier significantly reduced the proportions of embryos developing to 2-cell, 4-cell to morulae, and prevented any embryos developing to blastocysts, but increased the number of zygotes and abnormal embryos (Tab. III and Figs. 1C and 1F). Moreover, there were more 2-cell embryos at 85–90 h after mating of heated males (Tab. III).

3.4. The effect of paternal heat stress 35 days prior to mating on the in vivo development of mouse embryos

Paternal heat stress 35 days prior to mating significantly increased the number of zygotes, but reduced the proportion of embryos developing to the 2-cell stage at 34–39 h after mating (Tab. IV). Furthermore, while there was no difference in the total number of blastocysts (B + EB), the proportion of embryos developing to blastocysts (B) was higher and fewer of those developed to expanded blastocysts (EB) in H35 than in C35 (Tab. IV).

3.5. The effect of paternal heat stress 7 and 35 days prior to mating on the number of TE and ICM in the blastocysts

There were significantly fewer total cells in the embryos sired by heat-stressed males on 7 days after heating, and in the number of cells in the trophectoderm and the inner cell mass, but, no change in the ICM/TE ratio (Tab. V). However, these differences were not significant in the embryos sired 35 days after heating (Tab. V). No blastocysts were available in the H21 group.

4. DISCUSSION

The results of this study support previous reports which have indicated that both the whole body heating and the local heating of the scrotum decreased sperm concentration, motility and viability, but increased the number of abnormal spermatozoa in the mouse [32, 33], bull [34–36], ram [12, 37] and boar [38, 39].

The results of this study also confirmed previous reports which have shown that when spermatozoa are subjected to an elevated temperature in the uterus or in vitro or when males are exposed to high ambient temperatures, the in vivo developmental rates of preimplantation embryos are reduced in the rabbit [10, 11] and in the mouse [7, 40]. The present finding that the number of 4-cell embryos to morulae 61–65 h after mating was significantly lower in the experimental groups at 7 and 21 days after heat stress, with a higher proportion of the embryos at the zygote or 2-cell stages was different from that of previous studies which suggested that paternal heat stress significantly increases the number of 4-cell embryos [7]. This discrepancy could be attributed to the different mouse strains used, heat-stress conditions and the time of collecting embryos. However, the decrease in the number of blastocysts 85–90 h after mating is in accord with earlier results [40]. Furthermore, a common feature in this experiment and previous studies [7, 18, 41] is that the heat-stressed male can reduce the developmental rate of preimplantation embryos and increase the number of retarded embryos.

Spermatozoa ejaculated on 7 days after heating would have been in the epididymis at the time of heating [41, 42]. Although the epididymis has previously been reported to be resistant to heat [43], heat stress could disturb epididymal maturation of the spermatozoa because heat stress has been shown to damage the chromatin structure of sperm in the epididymis of the mouse [44] and the bull [45]. The results of this study have shown that heat stress significantly reduced sperm concentration, motility, viability, but increased the number of abnormal spermatozoa. A reduction in sperm quality resulted in a decrease of the developmental rate of

Table III. The effect of paternal heat stress 21 days prior to mating on the in vivo development of embryos.

Time of embryos recovered after mating (h)	14–16		34–39		61–65		85–90	
Groups	C21	H21	C21	H21	C21	H21	C21	H21
No. females	4	5	5	6	5	5	6	7
No. corpora lutea	10.8 ± 1.71	9.8 ± 1.30	9.2 ± 1.48	8.7 ± 1.21	8.0 ± 1.22	8.6 ± 1.82	7.7 ± 1.37	9.0 ± 2.16
Recovered embryos	10.8 ± 1.71	9.2 ± 1.79	9.0 ± 1.22	8.3 ± 1.21	7.8 ± 1.48	8.2 ± 2.59	7.3 ± 1.03	8.3 ± 1.98
Zygotes	10.8 ± 1.71	8.8 ± 1.79	0.2 ± 0.45	3.3 ± 3.20***	0.0 ± 0.00	4.2 ± 3.35***	0.0 ± 0.00	1.6 ± 0.77***
2-C[a]			8.4 ± 1.95	4.5 ± 3.21***	0.0 ± 0.00	0.6 ± 0.89	0.0 ± 0.00	1.1 ± 0.90**
4-C to M					7.8 ± 1.48	0.2 ± 0.45***	2.0 ± 2.53	0.1 ± 0.38***
B							5.7 ± 2.73	0.0 ± 0.00***
Abnormal			0.6 ± 0.89	0.5 ± 0.84	0.0 ± 0.00	3.2 ± 1.64***	0.0 ± 0.00	5.4 ± 2.36***
Unfertilised	0.0 ± 0.00	0.4 ± 0.55						

[a] C, M, B, abnormal and unfertilised are the same as in Table II.
The values given are mean ± SEM. The values with ** and *** superscripts differ significantly ($P < 0.01$–0.001) between the heat-sired group (H21) and controls (C21) for embryo development (ANOVA analysis).

Table IV. The effect of paternal heat stress 35 days prior to mating on the in vivo development of embryos.

Time of embryos recovered after mating (h)	14–16		34–39		61–65		85–90	
Groups	C35	H35	C35	H35	C35	H35	C35	H35
No. females	4	4	4	5	4	4	6	5
No. corpora lutea	11.0 ± 2.16	10.8 ± 0.96	8.3 ± 0.96	8.8 ± 2.17	7.5 ± 1.29	7.3 ± 1.89	9.5 ± 1.38	8.6 ± 1.14
Recovered embryos	11.0 ± 2.16	10.3 ± 0.50	8.3 ± 0.96	8.4 ± 2.07	6.3 ± 1.26	6.3 ± 2.22	9.5 ± 1.38	8.4 ± 1.14
Zygotes	9.5 ± 0.58	10.3 ± 0.50	0.0 ± 0.00	2.4 ± 2.07***	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
2-C[a]			8.0 ± 0.82	5.6 ± 4.28***				
4-C to M					5.8 ± 0.96	6.3 ± 2.22	1.0 ± 1.55	0.6 ± 0.89
B							3.5 ± 3.21	5.6 ± 4.04**
EB							5.0 ± 4.73	2.2 ± 2.20**
B+EB							8.5 ± 2.88	7.8 ± 1.64
Abnormal	1.5 ± 1.73	0.0 ± 0.00	0.3 ± 0.50	0.4 ± 0.89	0.3 ± 0.50	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

[a] C, M, B and abnormal as defined in Table II; EB: expanded blastocysts.
The values given are mean ± SEM. The values with ** and *** superscripts differ significantly ($P < 0.01$–0.001) between the heat-sired group (H35) and controls (C35) for embryo development (ANOVA analysis).

Table V. The number of cells in blastocysts sired either 7 or 35 days after heating.

Sire	No. B[a]	ICM	TE	Total No. TE + ICM	Ratio (ICM/TE)
Control (C7)	20	18.2 ± 2.92	25.2 ± 6.17	43.8 ± 7.08	0.75 ± 0.19
Heated (H7)	20	14.8 ± 5.30*	19.0 ± 6.27**	33.7 ± 10.58***	0.82 ± 0.33
Control (C35)	21	17.1 ± 4.81	26.3 ± 8.35	43.5 ± 12.65	0.66 ± 0.12
Heated (H35)	17	16.4 ± 4.37	22.9 ± 5.37	39.4 ± 8.68	0.73 ± 0.17

[a] B: blastocysts, ICM: inner cell mass, TE: trophectoderm.
The values given in ratio are the sum of the addition of ICM/TE ratio in each embryo divided by the total number of blastocysts used. Values with *, ** and *** superscripts differ significantly ($P < 0.02$, 0.005 and 0.001) between the heat-sired group (H7) and controls (C7) for the number of ICM and TE cells (ANOVA analysis).

preimplantaion embryos arisen from the epididymal sperm of heat-stressed males. This is in agreement with an increased mortality of embryos produced with immature, epididymal spermatozoa collected from rams during scrotal insulation [12].

Paternal heat stress 7 days prior to mating reduced the number of TE and ICM cells in the blastocyst although the ratio of ICM/TE was not different. A reduction of TE and ICM in the blastocyst could decrease its autocrine capacity which could be extremely important in any communication between the conceptus and the uterus. This could directly affect the subsequent implantation and pregnancy and it is relevant that paternal heat stress has been reported to reduce implantation rates [7, 40] and fetus weights and litter sizes [34, 46] in the mouse, and pregnancy rates in sheep [12], rats [18], pigs [6] and humans [24].

Twenty-one days after heating, there are significant reductions in the developmental rate of 2-cell embryos and an increase in the number of abnormal embryos. Additionally, no embryos developed into blastocysts when sired 21 days after heating. The increase of abnormal embryos in this study was similar to previous results published for the rat at 15 days after heating [18], and with the reduced implantation rate reported in the mouse at 21 days after heating [8]. A model proposed by Haig and Westoby [47] predicts that paternal imprinted genes will promote growth and maternal genes will inhibit growth. A paternal imprinted gene encoding U2afbp-rs, an RNA processing protein is transiently transcribed at the 2-cell stage in the mouse [48]. One possibility is that heat stress could impair and/or disturb the paternal imprinted genes during spermatogenesis, thus inhibiting subsequent embryo development. Furthermore, the higher proportion of 2-cell embryos 85–90 h after mating could be that endogenous markers of zygotic gene activation including a transcription requiring complex (TRC) [49–51], HSP70.1 [52–54] and a translation initiation factor (eIF-4C) [55] might either not be transcribed transiently and/or be abnormally transcribed during the 2-cell stage. These findings merit further investigation.

Spermatozoa ejaculated 21 days after heating would have been pachytene and diplotene spermatocytes at the time of heating [42, 43] and these cells have been previously reported to be seriously damaged by heat exposure [45, 56, 57] and this study. Apparently this damage can extend to an effect on the development of preimplantation embryos.

Paternal heat stress 21 and 35 days prior to mating also increased the number of zygotes at 34–39 h, 61–65 h and 85–90 h after mating, compared with the control groups. This may be because paternal heat stress could

damage nuclear remodelling and pronuclear formation [58, 59], inhibit the first round of DNA replication [60] and/or impair the regulatory balance between cell death genes and cell survival genes [61]. Furthermore, paternal heat stress 35 days earlier reduced the number of 2-cell embryos at 34–39 h after mating and expanded blastocysts 85–90 h after mating. Since sperm ejaculated 35 days after heating would have been spermatogonia at the time of heating, spermatogonia in the testis are also damaged by heat insult because heat stress reduced sperm concentration and motility and increased the number of abnormal spermatozoa of male mice. This was in agreement with a decreased sperm concentration and the increased number of abnormal spermatozoa recovered from the cauda epididymis of male mice, 35 days after exposure to a 40 °C water bath for 2 h [32].

In conclusion, the present results further demonstrated that embryo quality is related to sperm quality. These results suggest that heat stress damaged germ cells from both the epididymis and the testis, and significantly damaged spermatocytes, and possibly also spermatogonia because there were low sperm concentration, motility, viability and high abnormality in male mice after the treatment. A reduction in sperm quality after paternal heat stress leads to a decrease in the development rate of preimplantation embryos, significantly reducing the number of 2-cell embryos, 4-cell to morula and blastocysts.

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