EFFECTS OF INCREASED AMBIENT TEMPERATURE DURING IVM AND/OR IVF ON THE IN VITRO DEVELOPMENT OF BOVINE ZYGOTES

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SUMMARY

Previous research by this group (2003) has demonstrated that heat stress during in vitro culture (IVC) significantly increased early embryo mortality. The experiments reported here examine the effects of heat treatment (HT) during in vitro maturation (IVM) and during in vitro fertilization (IVF). One 24hr cycle of HT entailed a series of 0.5^{o}C incubator temperature increases from 39^{o}C to 39.5^{o}C for 2hrs, to 40^{o}C for 2 hrs, to 40.5^{o}C for 4 hours, 41^{o}C for 4hrs, 40.5^{o}C for 6 hours and 40^{o}C for 6hrs. This cycle mimics rectal temperatures recorded in high producing, grain fed dairy cows in hot climates. Experiment 1 studied the effects of one cycle of heat-treatment during IVF on the rate of cleavage of in vitro matured presumptive zygotes. Total cleavage rate in the HT group (37.8%) was lower than that of the control group (54.6%, $P<0.05$). Experiment 2 repeated the HT of experiment 1 but preceded it with a cycle of HT during IVM. The total cleavage rates for control and heat treatment groups were 75.5% and 37.9% respectively with a significant difference of $P<0.001$ identified. Experiment 3 examined the rates of embryonic development to ≥8-cell stage (after 72 hours IVC) and to morula or blastocyst (M/B) stage (after 144 hours IVC) following HT of the oocyte groups during the preceding IVM or IVF. Rates of development to ≥8-cell stage (at 72 hours IVC) and to M/B stage (after 144 hours IVC) for the control group were 27.5% and 35.8%. Those of IVM-only HT and IVF-only HT groups were 13.8% and 14.6%, and 8.6% and 14.3%, respectively. Both

groups of heat treated embryos developed at significantly ($p<0.05$) lower rates than did the control group. These results suggest that hyperthermia during oocyte maturation and/or fertilization adversely affects oocyte maturation and fertilization rates and retards further embryonic development.

Key words: heat treatment, hyperthermia, bovine, in vitro fertilization, in vitro maturation

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INTRODUCTION

Maximum productive output is constrained in hot climates by heat load from hot ambient conditions and by heat arising from metabolic processes, particularly in high producing, high concentrate-fed dairy cows and particularly where shade and spray cooling is inadequate (Hall, 2000). Where cattle are exposed to a high temperature environment during ovulation, the oocyte, attached cumulus cells and sperm are all subsequently subjected to high oviductal temperatures. Hirao & Yanagimachi (1978) reported negative effects on sperm-egg fusion and on post-fusion events due to high ambient temperatures. Cortical granule exocytosis, resumption of meiosis and pronuclear development are considered to be temperature dependent due to the series of intricate reactions occurring within the ovum cytoplasm; best performance of these reactions is likely to occur at the 39^{o}C core body temperature of the bovine. Putney et al. (1988) reported that the occurrence of high ambient temperatures (16 hours at 30°C and 8 hours at 42°C) between 30 hours after the beginning of oestrus (Day 1) to the seventh day after oestrus increased the number of abnormal and retarded embryos in artificially inseminated superovulated heifers. The following year (1989), they showed that high temperature treatment

between the onset of oestrus and insemination in superovulated dairy heifers decreased the number of normally developed embryos recovered on day 7 after oestrus. Ealy et al. (1993) exposed superovulated Holstein cows to high ambient temperatures on day 1, 3, 5 or 7 of pregnancy (day 0=oestrus) and examined the embryo survival rates at day 8 post-insemination. The respective proportions of normally developed transferable quality embryos were 55.0%, 68.0%, 65.0% and 88.9%. The results suggested that recently inseminated oocytes were most susceptible to maternal heat stress. In vitro, Lenz et al. (1983) reported that an ambient temperature at oocyte fertilization of 39°C produced a higher fertilization rate than did incubation at 35, 37 or 41°C. The following study was designed to examine the direct effects of high ambient temperature preceding, and/or at IVF through subsequent rates of zygote cleavage and embryo development. A sequence of incubator temperature alterations (Table 1) mimicked the recorded change in rectal temperatures of lactating grain fed dairy cows during hot seasons in south-east Queensland, Australia (Davison, et al., 1996).

MATERIALS AND METHODS

In vitro embryo production.

The in vitro embryo production process was as described by Sugiyama et al. (2003), differing only in the stage of the heat treatment cycle. Briefly, immature cumulus-oocyte complexes (COCs) were collected from abattoir-derived ovaries and those with ≥3 layers of cumulus cells were selected, washed and 10-12 were transferred into 45uL droplets of TCM-199 maturation medium (ICN Pharmaceuticals) containing 10% fetal calf serum (CSL Ltd., Parkville, Australia), 0.1 mg/mL Gentamycin, 2.2 μg/mL Pyruvate, 5.0 μg/mL Lutropin and 2.0 μg/mL Folltropin (Vetrepharm Pty Ltd., Melbourne, Australia). Maturation occurred under oil for 24 hours at 39°C, 100% humidity and 5% CO_2 in air. The matured COCs were graded

(Downs, 1989; Buccione et al., 1990) and those exhibiting expansion of at least one or two cumulus layers were selected to undergo IVF.

One 0.25 mL straw of frozen semen (same bull for all inseminations) was thawed and combined with 1 mL of 37°C Sperm Talp medium (Parrish *et al*., 1988) which was ten layered onto a Percoll (Pharmacia Biotech AB, Uppsala, Sweden) 90/70/50% gradient and centrifuged at 600×g for 20 minutes. The sperm pellet was transferred into 5 mL Fert Talp (Parrish et al., 1988) that included 20 μg/mL heparin (Sigma Chemical Co.) that was centrifuged at 300×g for 5 minutes to produce the final inseminate. Insemination concentration was 2 x 10^6 per droplet. Ten to fifteen selected COCs were washed and transferred into each 35uL droplet of Fert Talp (with 20 μg/mL heparin) under oil. Following insemination, they were placed into a Modular Incubator Chambers (MIC; Billups-Rothenberg, Inc. U.S.A.) and incubated in a β-gas (5%CO_2, 6.7%O_2, and 88.3% N_2; BOC Gases Ltd., Brisbane, Australia) atmosphere and 100 % humidity. Following 24 hours IVF, presumptive zygotes were washed, vortexed, transferred into 45uL of synthetic oviductal fluid medium (SOFM; Gardner et al., 1994) under oil and returned to the MIC culture environment.

Experiment I: Effects of Heat Treatment During IVF on Cleavage Rates of In Vitro Matured COCs

The matured COCs were equally divided into control and heat treatment (HT) groups, placed into separate β-gas supplied MICs and transferred into separate incubators for 24 hours. The control group was incubated at 39°C whilst the HT group was placed into a heat treatment incubator for 24 hours. The temperature of this incubator was manually adjusted over that period as per the regime outlined in Table 1. After IVF, the control and heat treated oocytes were separately processed for IVC as described in Sugiyama et al. (2003). Following 24 hours

of IVC, the proportion of presumptive zygotes with at least 2 equal cell divisions was calculated. This experiment was repeated 6 times.

Experiment II: Effects of Heat Treatment During IVM and IVF on Cleavage Rates of Oocytes

Immature COCs were collected and allocated to control and HT groups. The control group COCs underwent IVM and IVF at 39°C as described earlier. The HT group of COCs were similarly prepared but instead subjected to one 24 hour cycle of heat treatment (Table 1) during IVM and again during IVF. Both groups of presumptive zygotes were then removed from the fertilization medium and the surrounding cumulus cells were removed by vortexing. Each group of presumptive zygotes was washed, transferred to separate droplets of SOFM and incubated in a single MIC in β-gas, 100 % humidity atmosphere at 39°C for 24 hours. After 24 hours of IVC, the cleavage development rates of each group of presumptive zygotes were determined by light microscopy as per Experiment I. This experiment was repeated 5 times.

Experiment III: Effects of Heat Treatment of Oocytes During IVM or IVF on Rates of Cleavage and Early Embryo Development

Immature COCs were allocated to three equal size groups: control group, heat treatment during IVM (HT-IVM) and heat treatment during IVF (HT-IVF). The control group and HT-IVF group COCs were matured in vitro under control conditions for 24 hours as described previously. The HT-IVM group underwent one cycle of HT (Table 1) during IVM. After IVM, the control and HT-IVM groups were fertilized for 24 hours in vitro under control conditions, as described previously. The HT-IVF group was similarly prepared and fertilized in vitro but was subjected to one cycle (24 hours) of HT during IVF. After IVF, each group's COCs were removed from the fertilization medium and denuded. The presumptive zygotes from each group underwent IVC under control conditions for 72 hours after which they were

examined for evidence of cleavage and the number of embryos at ≥ 8-cell stage recorded. All zygotes from each group were placed into fresh SOFM and cultured for a further 72 hours. At 144 hours IVC, each group's embryos were examined and the number of morulae and blastocysts were recorded. This experiment was repeated 5 times.

Statistical analysis

Proportional differences between groups of presumptive zygotes undergoing cell cleavage (≥2-cells) and developing to ≥8-cell, morulae or blastocyst stages were assessed using the Chi squared test. Probability (P) values less than 0.05 were considered to be the minimal acceptable levels for statistical significance.

RESULTS

Experiment I

After 24 hours of IVC, the total cleavage rate of oocytes subjected to one cycle of HT during IVF was significantly ($P<0.05$) lower (37.8%; n=484) than that of the control group (54.6%; n=496). Overall, the percentage of 2-cell stage embryos in the control group (17.7%) was similar to that in the HT group (19.2%), however the percentage of 4-cell stage embryos in the control (32.5%) and treatment (17.6%) groups were significantly different ($P<0.001$). A much lower percentage of 1-cell or degenerate embryos/ova was seen in the control group (45.5%) than in the HT group (62.2%) ($P<0.05$). Heat treatment during IVF negatively affected subsequent embryo development.

Experiment II

Oocytes subjected to HT during IVM and IVF produced significantly lower ($P<0.05$) total cleavage rates after 24 hours of IVC (37.9%; n=393) than did the control group (75.5%; n=380). The percentage of 2-cell stage embryos in the control group (15.3%) was not statistically different from the treatment group (18.9%), but the percentages of 4-cell stage embryos in the control group (55.3%) were significantly greater ($P<0.001$) than that of HT group (18.8%). The control group (24.5%) produced significantly fewer ($P<0.05$) 1-cell or degenerate embryos or ova than did the HT group (62.1%). Thus, heat treatment during IVM and IVF impaired embryo viability.

Experiment III

Table 2 documents the overall percentages of 2-cell, 4-cell and ≥8-cell stage embryos after 72 hours of IVC in the control (n=345), HT-IVM (n=347) and HT-IVF (n=326) groups. There was a significant difference in the development rate of ≥8 cell stage embryos between the HT-IVM and HT-IVF groups ($P<0.05$), and between these treatment groups and the control group ($P<0.005$). The rates of progression to morula or blastocyst stages following 144 hours of IVC were similar for the HT-IVM group (14.6%) and the HT-IVF group (14.3%) but both were significantly lower ($P<0.001$) than that of the control group (35.8%).

DISCUSSION

The present in vitro experiments were designed to examine the effects of increased ambient temperatures on high production dairy cattle during the oestrus and fertilization periods. The heat treatment applied to immature oocytes, sperm, inseminated oocytes and fertilized oocytes represented the pattern of rectal temperatures recorded in live cattle in hot seasons in south-east Queensland (Davison, et al., 1996; Table 1).

Similar comparative in vitro studies by Lenz et al. (1983) and Edwards and Hansen (1996) used a peak IVM incubation temperature of 41°C for 25 hours and 12 hours, respectively. Lenz et al., (1983) subsequently reported a significantly ($p<0.0001$) reduced rate of nuclear maturation. They also demonstrated that an elevated temperature of 40°C was sufficient to negatively affect the sperm acrosome and acrosome reaction causing cell death. A subsequent reduction ($p<0.01$) in the fertilisation rate following IVF at 41°C was not unexpected. Rivera & Hansen (2001) showed that bovine oocytes fertilized at 41°C for 8 hours had lower cleavage rates than did oocytes fertilized at 38.5°C in-vitro; however, incubation at 40°C during IVF had no effect on cleavage rate and tended to increase the rate of oocytes forming blastocysts compared with oocytes fertilized at 38.5°C.

This study mimicked rectal temperatures recorded during very hot mid-summer days in south-east Queensland. The high ambient temperatures combined with the body heat produced from metabolic processes (typical of large lactating concentrate-fed Holstein Friesian dairy cows) to produce gradual core temperature increases to 41°C for up to 4 hours before gradually returning to normal. The inertia in response time of core body temperature to changes in ambient temperature saw the rectal temperature at over 40°C for up to 14 hours in some cows.

Based on cell cleavage, Experiments I and III recorded poor fertilization rates compared to the control group confirming that not only do high ambient temperatures during IVF impair fertilization, but that an exposure to a maximum of 41°C for only 4 hours is sufficient to adversely affect the outcome of IVF.

The final experiment demonstrated how HT of presumptive zygotes during IVF-only (HT-IVF; 8.6%) was significantly lower ($P<0.05$) than that of presumptive zygotes exposed to heat treatment during IVM-only (HT-IVM; 13.8%), and development of both HT groups was significantly lower ($P<0.05$) than that of the control group (27.5%). Whilst subsequent rates of morula/blastocyst production did not differ between HT groups, their development was

significantly (P>0.001) impaired compared to the non-HT control group. Edwards & Hansen (1997) reported that embryonic heat resistance decreased over the period from oocyte insemination to development to the two-cell stage, but that this resistance gradually returned. Work described earlier by Ealy et al. (1993) supported the latter, noting the increased susceptibility that 2-cell embryos have to heat shock as compared to morula. This finding supports those of the current study that suggest hyperthermia during IVF is more detrimental to embryo development than it is during IVM. In Israel, Wolfenson, et al. (1988) demonstrated that conception rate of non-cooled Israeli Holstein dairy cows during summer was lower (17%) than that of cooled cows (59%) even if the maximum average body temperature of non-cooled cows was lower than 40°C (39.7°).

Edwards & Hansen (1996) further reported that the incubation of COCs at 41°C for the first 12 hours of IVM did not significantly affect the cleavage rate, but did decrease the rate of development to the blastocyst stage. Although the current study only utilised a 4 hour period of 41°C, the gradual increase to that peak temperature (Table 1) meant that embryos were exposed to temperatures of over 40°C for 14 hours and this proved sufficient to reduce the rates of both zygote cleavage and development to morula or blastocyst stage.

Collectively, the findings of the present study demonstrate that elevated temperatures characteristic of heat stress can compromise embryonic development when occurring during maturation and fertilization. The exact mechanisms for this are still not defined despite the work by Edwards & Hansen (1996, 1997) in the area of heat shock proteins (HSPs). Heat stress in immature/maturing oocytes appears to impair protein synthesis, there appears to be an inadequacy of appropriate HSPs, and it is thought that free radicals produced during heat stress can change the surface of the zona pellucida and cytoplasm (Edward & Hansen, 1996) impairing sperm penetration. Heat shock to oocytes peri-fertilisation could affect syngamy or the first step of cleavage, because these processes include microtubule and microfilament

assembly (Kim et al., 1997). It is also known that cytoskeletal elements break down and accumulate after heat shock treatment (Welch & Suhuan, 1985). Although heat treatment adversely affected IVM and IVF, some ova did mature, were fertilized and did develop normally. This suggests that bovine ova vary in their susceptibility to the effects of heat stress for undefined reasons; that acquisition of thermotolerance may be related to the capacity to synthesise HSPs in response to excessive heat.

Acknowledgments

We would like to thank Dr B Dobronov, the management and staff at AMH Dinmore abattoir for allowing us to collect cattle ovaries for our in vitro experiments.

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Table 1. Temperature regime of the incubator being used for heat treatment of oocytes over a 24 hour period.

Temperature of the incubator (°C)	Incubation time (hours)
39.5	2
40.0	2
40.5	4
41.0	4
40.5	6
40.0	6
	= 24 hours (one cycle)

290 Table 2. Effect of no heat treatment (control group) and of heat treatment during IVM (HT-IVM) or IVF (HT-IVF) on the number of presumptive zygotes developing to 1, 2, 4 and ≥8-cell stages after 72 hours of IVC and from ≥8-cell stage to morula or blastocyst (M/B) stage after 144 hours of IVC.

Stage of embryonic division	Control group (%)	HT-IVM group (%)	HT-IVF group (%)
1 cell or degenerate	158 (45.8)	195 (56.2)	217 (66.6)
2 cell	27 (7.8)	25 (7.2)	24 (7.4)
4 cell	65 (18.8)	79 (22.8)	57 (17.5)
≥8 cell	95 (27.5)[a]	48 (13.8)[b]	28 (8.6)[c]
Development of ≥8-cell embryos to M/B stage (%)	34/95 (35.8)[d]	7/48 (14.6)[e]	4/28 (14.3)[e]
Total number	345	347	326

[a] and [b,c] are significantly different ($p<0.05$)

295 [d] and [e] are significantly different ($p<0.001$)