Increased apoptosis in bovine blastocysts exposed to high levels of IGF1 is not associated with downregulation of the IGF1 receptor

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Abstract

The hypothesis that high concentrations of IGF1 can impair embryo development was investigated in a bovine *in vitro* model to reflect conditions in polycystic ovary syndrome (PCOS) patients. Embryos were either cultured in the absence or presence of a physiological (100 ng/ml) or supraphysiological (1000 ng/ml) IGF1 concentration. Cell allocation, apoptosis, transcript and protein expression of selected genes involved in apoptosis, glucose metabolism and the IGF system were analysed. Supraphysiological IGF1 concentration did not improve blastocyst formation over controls, but induced higher levels of apoptosis, decreased TP53 protein expression in the trophectoderm and increased the number of cells in the inner cell mass (ICM). The increase in ICM cells corresponded with an increase in IGF1 receptor (IGF1R) protein in the ICM. A small, but significant, percentage of blastocysts displayed a hypertrophic ICM, not observed in controls and virtually absent in embryos treated with physiological concentrations of IGF1. Physiological IGF1 concentrations increased total IGF1R protein expression and upregulated IGFBP3 transcripts leading to an increase in blastocyst formation with no effects on cell number or apoptosis. In conclusion, the results support the hypothesis of detrimental effects of supraphysiological IGF1 concentrations on early pregnancy. However, our results do not support the premise that increased apoptosis associated with high levels of IGF1 is mediated via downregulation of the IGF1R as previously found in preimplantation mouse embryos. This *in vitro* system with the bovine preimplantation embryo reflects critical features of fertility in PCOS patients and could thus serve as a useful model for in-depth mechanistic studies.

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Introduction

The insulin-like growth factor (IGF) system is a critical signalling mechanism disrupted by hyperinsulinaemia in conditions such as the polycystic ovary syndrome (PCOS; Essah *et al.* 2004). Hyperinsulinaemia has been suggested to be a risk factor for miscarriage in PCOS patients (van der Spuy & Dyer 2004, Cocksedge *et al.* 2008) and has been proposed as a main factor in the pathophysiology of early pregnancy loss in PCOS patients (Essah *et al.* 2004). The increased insulin levels reduce synthesis of IGF binding proteins (IGFBPs) such as IGFBP1, which in turn enhances bioactivity of IGF1 (Wang & Chard 1999). High concentrations of free IGF1 affect endometrial function with deteriorating effects on implantation (Lathi *et al.* 2002, Giudice 2006). Exposure of rodent embryos to increased concentrations of IGF1 *in vivo* or *in vitro* resulted in abnormal preimplantation embryo development (Katagiri *et al.* 1996, 1997). These findings led to the hypothesis that high IGF1

concentrations are partially responsible for the early pregnancy losses observed in PCOS patients (Chi *et al.* 2000). To test this hypothesis, murine embryos were cultured *in vitro* in the presence of IGF1 concentrations ranging from 950 to 1500 ng/ml. These high levels of IGF1 induced apoptosis in blastocysts via downregulation of the IGF1 receptor (IGF1R; Chi *et al.* 2000, Eng *et al.* 2007), which resulted in increased resorption rates after transfer to recipients (Pinto *et al.* 2002, Eng *et al.* 2007).

Current data indicate that high IGF1 concentrations downregulate the IGF1R in mouse embryos causing a decrease in glucose uptake associated with reduced activation of the AMP kinase (Chi *et al.* 2000, Pinto *et al.* 2002, Eng *et al.* 2007, Louden *et al.* 2008). IGF1-induced apoptosis in murine blastocysts is dependent on expression of BAX, TP53 and caspases (1, 3 and 8) (Chi *et al.* 2000, Moley *et al.* 2005). In contrast, downregulation of IGF1R has been reported in bovine embryos exposed to IGF1 at physiological

concentrations (100 ng/ml; Prella *et al.* 2001, Block *et al.* 2008). In fact, transfer of *in vitro*-produced bovine embryos treated with 100 ng/ml IGF1 improved both pregnancy and calving rates in lactating cows suffering from heat stress (Block *et al.* 2003, Block & Hansen 2007). An IGF1 dose-dependent decrease in IGF1R expression was reported in mouse embryos (Chi *et al.* 2000) and whether this occurs in bovine embryos is unknown at present. With the exception of one report (Inzunza *et al.* 2010), the general consensus is that mouse embryos start expressing IGF1R at the eight-cell stage (Smith *et al.* 1993, Stojanov & O'Neill 2001, Markham & Kaye 2003), whereas in human, porcine and bovine embryos IGF1R is expressed throughout the preimplantation period (Watson *et al.* 1992, Lighten *et al.* 1997, Yaseen *et al.* 2001, Kim *et al.* 2005). This highlights the importance of using alternative animal models to laboratory mice for investigations on human-related endocrine pathologies. Since *Bos taurus* is a good model species for early human embryo development (Ménézo & Héribel 2002, Baumann *et al.* 2007, Kues *et al.* 2008, Velazquez 2008), bovine *in vitro* embryo production (IVP) could provide a good experimental model system for unravelling mechanisms involved in the putative impaired development of embryos exposed to high concentrations of IGF1. A cross-species microarray hybridisation study revealed that gene expression profiles in bovine and human blastocysts were to a large extent identical (Adjaye *et al.* 2007). Similarly, the transcription profiles during maternal–zygotic transition are similar between the two species (Xie *et al.* 2010). Furthermore, the amino acid sequence of bovine IGF1 is identical to that of human IGF1 (Honegger & Humbel 1986, Francis *et al.* 1988). In both the species, supplementation with physiological concentrations of human recombinant IGF1 can exert positive effects on *in vitro* preimplantation embryo development (Lighten *et al.* 1998, Spanos *et al.* 2000, Byrne *et al.* 2002, Makarevich & Markkula 2002).

IGF1 has anti-apoptotic activities, and exerts strong mitogenic effects on several mammalian cell types (Benito *et al.* 1996) including blastomeres from preimplantation embryos. For instance, early embryos exposed to physiological concentrations of IGF1 (50–150 ng/ml) showed an increased number of cells in the resulting blastocysts in several mammalian species including mice (Lin *et al.* 2003, Głabowski *et al.* 2005), gerbil (Yoshida *et al.* 2009), cattle (Byrne *et al.* 2002, Makarevich & Markkula 2002, Sirisathien *et al.* 2003, Jousan & Hansen 2007), buffaloes (Narula *et al.* 1996), pigs (Kim *et al.* 2005, 2006), rabbits (Herrler *et al.* 1998) and humans (Lighten *et al.* 1998). Physiological (100 ng/ml) and supraphysiological (1000 ng/ml) concentrations of IGF1 increased the total cell number (TCN) in mice embryos in a dose-dependent manner (Głabowski *et al.* 2005). It is unknown whether the IGF1-induced increase in the number of cells in murine embryos is associated

with an elevated apoptotic index (Chi *et al.* 2000). The effects of high concentrations of IGF1 on cell allocation in preimplantation embryos, which is a critical factor for a successful pregnancy, have not yet been investigated. Unravelling the mechanisms by which high concentrations of IGF1 interfere with normal embryonic development could be beneficial for the development of more efficient therapies in PCOS patients.

The goal of this study was to determine the effects of supraphysiological concentrations of IGF1 on preimplantation bovine embryos. We evaluated cellular and molecular characteristics of *in vitro*-produced bovine blastocysts exposed to physiological or supraphysiological concentrations of IGF1 from the zygote stage onwards. The parameters analysed at the blastocyst stage included cell allocation, frequency of apoptosis, protein expression of the IGF1R and TP53 gene and transcript abundance of selected genes involved in apoptosis, glucose metabolism and the IGF system.

Results

Preimplantation embryo development

Cleavage rates were not affected by IGF1 supplementation. However, the total rate of blastocyst formation was increased significantly by 100 ng/ml IGF1 compared with controls ($P=0.005$) and 1000 ng/ml IGF1 ($P=0.035$; Table 1). The 100 ng/ml IGF1 group had fewer degenerated embryos than the control ($P=0.014$) and the supraphysiological IGF1 group ($P=0.018$). The rate of blastocyst formation and degenerated embryos did not differ significantly between the control and the 1000 ng/ml IGF1 group (Table 1). The proportion of replicates in which blastocyst formation was increased over controls was higher for 100 ng/ml IGF1 than for 1000 ng/ml IGF1 (80.6 vs 51.6%, $P=0.32$).

Differential cell staining and detection of apoptosis by TUNEL

The number of cells in the inner cell mass (ICM) was similar in the control and the 100 ng/ml IGF1 group. The supraphysiological IGF1 concentration increased the number of ICM cells over controls ($P=0.005$) and the 100 ng/ml IGF1 group ($P=0.003$). The number of cells in the trophectoderm (TE) did not differ between the three groups (Table 2). Mean values for ratio of TE to ICM cells and the proportion of ICM to the TCN (ICM/TCN) were not affected by IGF1 treatment (Table 2). However, a significant percentage of blastocysts in the 1000 ng/ml IGF1 group displayed an increased ICM/TCN proportion (>40) compared with the control ($P=0.006$) and the 100 ng/ml IGF1 group ($P=0.006$; Fig. 1). The control ($P=0.001$) and the 100 ng/ml IGF1 group ($P=0.006$) had a higher percentage of blastocysts with a 20–40 ICM/TCN proportion than the supraphysiological IGF1 group.

Table 1 Effects of different insulin-like growth factor 1 (IGF1) concentrations on the *in vitro* development of bovine embryos.

Parameters	Control (n=1200) ^a	100 ng/ml IGF1 (n=1200) ^a	1000 ng/ml IGF1 (n=1200) ^a
	Mean ± S.E.M. – median (Q1–Q3) (n) ^b	Mean ± S.E.M. – median (Q1–Q3) (n) ^b	Mean ± S.E.M. – median (Q1–Q3) (n) ^b
Cleavage ^c (%)	55.9 ± 1.9 – 57.1 (48.5–62.8) (679)	58.4 ± 2.0 – 60.0 (54.2–63.3) (707)	58.6 ± 1.5 – 59.1 (51.9–64.0) (707)
Blastocysts (%)	7.9 ± 1.1 – 8.1 (2.5–13.6) (96)	9.6 ± 1.2 – 8.5 (5.0–14.2) (115)	7.1 ± 1.1 – 6.1 (0.5–11.1) (87)
Expanded blastocysts (%)	15.7 ± 1.1 – 16.6 (11.4–19.5) (194)	18.1 ± 0.7 – 17.1 (16.3–20.3) (219)	17.1 ± 1.3 – 18.3 (12.4–22.8) (208)
Hatching blastocysts (%)	2.6 ± 0.4 – 2.8 (0.0–4.2) (31)	2.6 ± 0.4 – 2.8 (0.0–4.4) (32)	2.1 ± 0.4 – 0.0 (0.0–5.0) (27)
Hatched blastocysts (%)	2.5 ± 0.5 – 2.3 (0.0–4.4) (29)	4.5 ± 0.8 – 2.8 (0.0–6.7) (54)	3.8 ± 0.6 – 2.8 (0.5–5.7) (47)
Total blastocysts (%)	28.9 ± 1.5* – 28.5 (23.5–34.2) (350)	34.9 ± 1.4 [†] – 32.6 (30.6–40.0) (420)	30.4 ± 1.5* – 31.4 (24.6–37.0) (369)
Degenerated embryos ^d (%)	47.5 ± 2.3* – 47.6 (39.4–58.2) (329)	38.5 ± 2.7 [†] – 42.1 (27.3–51.1) (287)	47.2 ± 2.7* – 46.6 (35.4–61.1) (338)

*[†]Values within rows with different superscripts differ significantly ($P \leq 0.05$).

^aPresumptive zygotes used in 31 IVP cycles. ^bNumber of embryos produced in each category. ^cEmbryos with four or more cells on day 3 (day 0 = IVF).

^dCalculated on the number of cleaved embryos.

The percentage of blastocysts showing an ICM/TCN proportion <20 was not statistically different between the three groups (Fig. 1).

The percentage of embryos with at least one apoptotic blastomere were higher in the 1000 ng/ml IGF1 (98.6%) group compared with the control (88%) group ($P=0.026$). There were no differences in this regard between the control and physiological IGF1 group (93.5%) or between the two IGF1 groups. No significant differences were found in any of the apoptotic parameters analysed between the controls and the 100 ng/ml IGF1 group. In contrast, embryos treated with 1000 ng/ml IGF1 showed more apoptotic cells in ICM ($P<0.001$) and TE ($P=0.004$) than embryos in the control and the 100 ng/ml IGF1 group (Table 2). This was reflected in a higher number of total apoptotic cells ($P<0.001$) and in an increased apoptotic index ($P<0.001$) in the supraphysiological group compared with the control and 100 ng/ml IGF1 groups (Table 2). The number of apoptotic cells was not dependent on the number of cells in any of the

groups as indicated by low coefficients of correlation (control: $r^2=0.0858$; 100 ng/ml IGF1: $r^2=0.0993$; 1000 ng/ml IGF1: $r^2=0.0047$). TCN was increased in 1000 ng/ml IGF1 compared with the control ($P=0.005$) and 100 ng/ml IGF1 groups ($P=0.01$) respectively. There were no differences in this regard between the control and the 100 ng/ml IGF1 group (Table 2).

mRNA transcript expression

Supplementation with IGF1 did not affect the relative abundance of transcripts for IGF1R, TP53 and SLC2A3. However, 100 ng/ml of IGF1 significantly increased transcript abundance of IGFBP3 ($P=0.006$; Fig. 2) and tended to increase the relative abundance of transcripts for SLC2A1 ($P=0.067$) and SLC2A8 ($P=0.06$) over that in embryos cultured in supraphysiological concentrations of IGF1. Transcript abundance for IGFBP3, SLC2A1 and SLC2A8 did not differ among the rest of the group combinations (i.e. control versus 100 ng/ml

Table 2 Effects of different insulin-like growth factor 1 (IGF1) concentrations on cell number, cell allocation and apoptosis in bovine blastocysts produced *in vitro*.

Parameters	Control	100 ng/ml IGF1	1000 ng/ml IGF1
	Mean ± S.E.M. – median (Q1–Q3) (n) ^a	Mean ± S.E.M. – median (Q1–Q3) (n) ^a	Mean ± S.E.M. – median (Q1–Q3) (n) ^a
Cell number in the ICM	32.3 ± 1.2* – 30.0 (26.0–40.0) (DCS=57)	32.5 ± 1.1* – 30.0 (25.0–38.0) (DCS=75)	39.0 ± 1.6 [†] – 36.5 (29.0–48.0) (DCS=66)
Cell number in the TE	89.6 ± 4.0 – 84.0 (69.5–113.2) (DCS=57)	96.7 ± 3.5 – 95.0 (75.0–118.5) (DCS=75)	102.3 ± 4.8 – 100.5 (64.0–133.0) (DCS=66)
Ratio TE:ICM	2.8 ± 0.1 – 2.6 (2.2–3.1) (DCS=57)	3.1 ± 0.1 – 2.9 (2.3–3.6) (DCS=75)	2.8 ± 0.1 – 2.5 (1.7–3.8) (DCS=66)
Proportion ICM/TCN (%)	27.2 ± 0.7 – 27.6 (24.2–30.6) (DCS=57)	25.8 ± 0.7 – 25.5 (21.3–29.8) (DCS=75)	28.8 ± 1.1 – 28.5 (20.6–36.0) (DCS=66)
Apoptotic cells in ICM	1.3 ± 0.2* – 0.0 (0.0–2.0) (TUNEL=76)	0.7 ± 0.1* – 0.0 (0.0–1.0) (TUNEL=77)	2.2 ± 0.3 [†] – 1.0 (0.0–4.0) (TUNEL=73)
Apoptotic cells in TE	3.2 ± 0.3* – 3.0 (1.0–5.0) (TUNEL=76)	3.8 ± 0.3* – 3.0 (1.0–5.0) (TUNEL=77)	5.4 ± 0.5 [†] – 4.0 (1.0–9.2) (TUNEL=73)
Total apoptotic cells	4.6 ± 0.4* – 3.5 (1.0–7.0) (TUNEL=76)	4.5 ± 0.4* – 4.0 (2.0–6.2) (TUNEL=77)	7.7 ± 0.6 [†] – 7.0 (3.7–12.0) (TUNEL=73)
Total apoptotic index (%)	3.0 ± 0.3* – 2.5 (0.9–4.5) (TUNEL=76)	2.9 ± 0.2* – 2.1 (1.4–3.6) (TUNEL=77)	4.8 ± 0.3 [†] – 5.0 (2.3–6.7) (TUNEL=73)
Total number of cells	146.0 ± 3.4* – 140.0 (109.2–180.7) (DCS–TUNEL–IF=179)	147.1 ± 3.3* – 135.0 (111.0–170.2) (DCS–TUNEL–IF=197)	161.4 ± 3.9 [†] – 151.0 (119.5–199.2) (DCS–TUNEL–IF=189)

ICM, inner cell mass; TE, trophoctoderm; TCN, total cell number. *[†]Values within rows with different superscripts differ significantly ($P \leq 0.05$).

^aNumber of embryos used for differential cell staining (DCS), terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) or immunofluorescence (IF).

Figure 1 Effect of different IGF1 concentrations on the percentage of *in vitro*-produced blastocysts displaying different inner cell mass (ICM)/total cell number (TCN) proportions. Within ICM/TCN categories, bars with different letters differ significantly ($P \leq 0.05$).

IGF1; control versus 1000 ng/ml IGF1). Physiological concentrations of IGF1 tended to increase the relative expression of AKT1 transcripts compared with controls ($P=0.059$), but not to 1000 ng/ml IGF1. Relative abundance of AKT1 did not differ between the control and the supraphysiological IGF1 group.

IGF1R immunofluorescence

The IGF1R was localised in both the ICM (ICM-positive) and TE in most of the blastocysts, predominantly in the cytoplasm (Fig. 3). However, a substantial proportion of blastocysts did not display IGF1R in the ICM (ICM-negative). The proportion of ICM-negative embryos did not differ significantly between the groups (control = 34.7%, 100 ng/ml IGF1 = 29.1% and 1000 ng/ml IGF1 = 50%). In ICM-positive embryos, IGF1R was less expressed in the ICM than in the TE (Fig. 4). Less ICM immunofluorescence in ICM-positive embryos and ICM-negative staining was also observed in hatched blastocysts, indicating that the presence of the zona pellucida did not affect the results. No differences in relative signal strength (RSS) were found between the groups when all embryos were analysed together (i.e. ICM-positive and ICM-negative blastocysts). A second statistical analysis carried out in ICM-positive embryos revealed increased RSS in the ICM of embryos treated with 1000 ng/ml IGF1 ($P=0.023$) compared with the control group (Fig. 4). No significant differences in TE immunofluorescence were observed between high IGF1 treated and control embryos. Embryos treated with 100 ng/ml IGF1 tended to have more IGF1R

immunofluorescence in the ICM ($P=0.076$) and showed a significant increase in TE immunofluorescence ($P=0.014$) over controls (Fig. 4). Total RSS values for IGF1R were higher for 100 ng/ml IGF1 ($P=0.008$) when compared with controls (Fig. 4). There were no significant differences in RSS values in IGF1 groups from ICM-positive embryos and between all groups in ICM-negative blastocysts. There was no correlation between the RSS values and the total number of nuclei in any of the groups.

TP53 immunofluorescence

The TP53 protein was observed in both the ICM and TE in most of the embryos (Fig. 3). Only a few blastocysts did not display TP53 in the ICM (control = 2, 100 ng/ml IGF1 = 2 and 1000 ng/ml IGF1 = 1). All embryos showed cytoplasmic localisation of TP53 protein. RSS values were

Figure 2 Relative transcript abundance (mean $\pm$ S.E.M.) of developmentally important genes in day-8 blastocysts cultured in the presence of different IGF1 concentrations from the zygote stage onwards. Bars with different superscripts within each gene transcript indicate a significant difference ($P \leq 0.05$).

Figure 3 Three-dimensional reconstruction of confocal laser microscopy images showing protein localisation of the IGF1R and TP53 in day-8 blastocysts cultured in the presence of different concentrations of IGF1. PI, propidium iodide. Note the lower expression of the IGF1R in the inner cell mass of the control group compared with the IGF1 groups and the lower expression of TP53 in the trophoderm in the supraphysiological IGF1 group (1000 ng/ml).

similar between the ICM and TE in the control group, whereas in the IGF1 groups TP53 immunofluorescence was less intense in the ICM (Fig. 5). RSS values of TP53 in the ICM were not affected by IGF1 treatment. TE RSS values of TP53 were not different between controls and physiological IGF1. However, embryos treated with 1000 ng/ml IGF1 had a decreased TP53 RSS in TE compared with the controls ($P=0.033$) and treatment with 100 ng/ml IGF1 ($P=0.048$). Supraphysiological IGF1 decreased total RSS compared with controls ($P=0.022$) but not when compared with the physiological IGF1 group (Fig. 5). Total p53 RSS values were not different between the control and physiological IGF1 group. Total nuclei were not correlated with RSS values in any of the groups.

Discussion

The novel finding of this study is that the increased apoptosis in bovine blastocysts induced by supraphysiological IGF1 concentrations was not associated with downregulation of the IGF1R, as previously found for

preimplantation murine embryos (Chi *et al.* 2000, Pinto *et al.* 2002, Eng *et al.* 2007). On the contrary, high levels of IGF1 increased IGF1R protein expression specifically in the ICM. Upregulation of IGF1R has been observed in bovine embryos cultured in the presence of 100 ng/ml Long R3 IGF1, which has a 1000-fold reduced affinity for IGF1Rs (Prelle *et al.* 2001). The differential regulation of IGF1R during exposure to high levels of IGF1 could involve, yet unknown, signalling networks in the bovine embryo. For instance, IGF1 regulates expression of transcription factors such as Kruppel-like factor 6 (KLF6) in a TP53-dependent manner (Bentov *et al.* 2008). Its protein expression levels seem to be directly related to the protein levels of IGF1R (Rubinstein *et al.* 2004). KLF6 has been identified in murine embryonic cells (Laub *et al.* 2001) and is involved in murine embryonic stem (ES) cell differentiation (Matsumoto *et al.* 2008). KLF6 activates genes involved in cell proliferation and survival such as members of the transforming growth factor β (TGFB) family, including the TGFB type III receptor (TGFB3; Friedman *et al.*

Figure 4 Relative signal strength (RSS) values (mean $\pm$ S.E.M.) of the IGF1R in day-8 blastocysts treated with different concentrations of IGF1 and displaying immunofluorescence in the inner cell mass (ICM) and the trophoctoderm (TE). Between groups, bars with different superscripts indicate a significant difference (ICM: a, b; TE: c, d; total RSS: A, B; $P \leq 0.05$). Within groups, the asterisk indicates significant differences between the ICM and the TE ($P \leq 0.05$).

2007). Accordingly, over-expression of TGFBR3 induces cellular apoptosis (Margulis *et al.* 2008). In this scenario, downregulation of the IGF1R would not be necessary to induce apoptosis. The TGFB signalling pathway is operative in both human (Adjaye *et al.* 2005) and bovine embryos (Huang *et al.* 2010). Interestingly, TGFB signalling pathway was associated with embryo demise, whereas TGFBR3 was upregulated in bovine embryos that did not complete the morula-to-blastocyst transition by day 8 after IVF (Huang *et al.* 2010).

Species-specific differences in embryonic IGF signalling could also account for the difference in regulation of IGF1R under high levels of IGF1 between mice and cattle. Gene expression of the IGF1 ligand has been detected from the zygote to the blastocyst stage in mouse embryos (Stojanov & O'Neill 2001) and immunolocalisation studies confirmed its presence, revealing a higher expression in the ICM than in the TE cells (Stojanov & O'Neill 2001). In contrast, early studies had reported the presence of mRNA for IGF1 ligand in preimplantation bovine embryos (Watson *et al.* 1992, Yoshida *et al.* 1998, Lonergan *et al.* 2000), but subsequent experiments did not confirm these observations (Yaseen *et al.* 2001, Bertolini *et al.* 2002, Ponsuksili *et al.* 2002, Moore *et al.* 2007, Warzych *et al.* 2007, Wang *et al.* 2009). This indicates an active autocrine IGF1 circuit in mice compared with cattle, similar to that of the ovary (Velazquez *et al.* 2008).

In rabbits (Herrler *et al.* 1997) and mice (Smith *et al.* 1993), the IGF1R is equally expressed in both the ICM and TE, although a recent study indicated that rabbit blastocysts expressed more IGF1R in the ICM than in the TE cells (Navarrete Santos *et al.* 2008). This differs from the low or lack of expression of IGF1R in the ICM of bovine blastocysts found in this study and by others (Wang *et al.* 2009). The lack of IGF1 production and

the preferential expression of the IGF1R in the TE have also been observed in human embryos (Hardy & Spanos 2002).

In this study, the higher ICM protein expression of the IGF1R observed in embryos treated with supraphysiological IGF1 concentrations corresponded with a specific increase in ICM cells. This is consistent with previous studies that showed a selective increase of cells in the ICM in bovine blastocysts (Sirisathien *et al.* 2003) and embryos from other species treated with IGF1, including humans (Lighten *et al.* 1998), pigs (Kim *et al.* 2005) and mice (Smith *et al.* 1993). The localised cell proliferation seems to be related to activation of the MAPK pathway in the ICM by IGF1 (Navarrete Santos *et al.* 2008). Nguyen *et al.* (2007) demonstrated that IGF1 can increase proliferation of mouse ES cells by stimulating protein synthesis via the MAPK pathway, similar to the situation observed in intact blastocysts (Navarrete Santos *et al.* 2008). Likewise, it has been found that IGF1R is co-localised with POU5F1 (Oct4) expression in human ES cells and plays a critical role for expansion of ES cell cultures as shown by the reduced cell number after IGF1R blocking (Bendall *et al.* 2007). The increase in IGF1R in the ICM was not translated into improved blastocyst formation, suggesting that IGF1 requires a simultaneous increase of its receptor in both cell compartments to exert beneficial effects upon bovine embryo development. Indeed, a more homogenous increase in IGF1R protein was detected in the group of embryos treated with physiological concentrations of IGF1, where increased blastocyst yields were found. This positive effect of IGF1 at physiological concentrations is probably related to improved glucose uptake via its own receptor (Pantaleon & Kaye 1996) and could be due in part to a better modulation of IGF1 binding via higher expression of IGBP3 observed in this and other studies (Prelle *et al.* 2001, Block *et al.* 2008).

Figure 5 Relative signal strength (RSS) values (mean $\pm$ S.E.M.) of TP53 in day-8 blastocysts treated with different concentrations of IGF1. ICM, inner cell mass; TE, trophoctoderm. Between groups, bars with different superscripts indicate a significant difference (TE: a, b; total RSS: A, B; $P \leq 0.05$). Within groups, the asterisk indicates significant differences between the ICM and the TE ($P \leq 0.05$).

The lower protein expression of TP53 in the supra-physiological IGF1 group was rather surprising since activation of TP53 is considered indicative of embryonic demise (Keim *et al.* 2001, Matwee *et al.* 2001). However, recent data suggest that TP53 does not act merely as a death signal, but rather as a modulator of damage responses to ensure successful development (Toyoshima 2009). It has been suggested that the primordial function of the *TP53* ancestor gene was to ensure fecundity and production of normal offspring and that its function as a tumour suppressor came later in evolution (Hu 2009). Accordingly, there is evidence indicating that expression of TP53 is essential for female fertility (Hu *et al.* 2007) and that active TP53-dependent cell death signalling is required to suppress erroneous replication of damaged DNA during the preimplantation period (Toyoshima 2009) and to avoid abnormalities during foetal development (Torchinsky & Toder 2010). In fact, high levels of TP53 are associated with regular cellular differentiation during murine embryogenesis (Schmid *et al.* 1991). From this perspective, we propose that the decrease in TP53 in embryos treated with high levels of IGF1 reflects a reduced capacity to eliminate unfit phenotypes, such as embryos with aberrant cell allocation.

Data from *in vivo*-produced bovine embryos indicate that an ICM/TCN proportion between 20 and 40% is within the normal range (Van Soom *et al.* 1997, Koo *et al.* 2002, Rho *et al.* 2007). In this study, a significant percentage of embryos treated with supraphysiological concentrations of IGF1 developed a high ICM/TCN proportion (>40%). Control blastocysts did not exhibit this phenotype and it was practically absent in embryos treated with physiological concentrations of IGF1. A high ICM/TCN proportion (41.3%) was found in experiments in which cysteine addition to the synthetic oviductal fluid (SOF) medium supplemented with foetal bovine serum increased apoptosis and reduced blastocyst formation and hatching rates (Van Soom *et al.* 2002). Furthermore, high ICM/TCN proportions (42–60%) have been reported in bovine somatic cell nuclear transfer (SCNT) embryos (Koo *et al.* 2002, Amarnath *et al.* 2004, Li *et al.* 2004, 2007, Oh *et al.* 2006). The aberrant cell allocation of bovine SCNT embryos has been suggested to be partially responsible for the high embryonic losses occurring during early pregnancy (first trimester) after embryo transfer (Koo *et al.* 2002). We presume that the increased apoptosis observed mainly in the TE and the relative reduction in TE cell number caused by the high ICM/TCN proportion can give rise to either implantation failures or a foetus with impaired placental function with detrimental effects on pregnancy outcome. This hypothesis needs to be tested in embryo transfer studies.

Our data also indicate that the majority of embryos can cope with high concentrations of IGF1 and develop with a normal cell allocation. In our static *in vitro* system, consumption and degradation of IGF1 occurs

without peptide renewal. In contrast, in a naturally occurring high IGF1 microenvironment such as with PCOS (Thierry van Dessel *et al.* 1999), the embryos are continuously exposed to abnormally high levels of IGF1, which may exacerbate the phenotype observed in our *in vitro* model (high apoptosis and hypertrophic ICM). Furthermore, given that oestrogens regulate to a great extent IGF1 production in the uterus (Velazquez *et al.* 2009), an increased paracrine action of IGF1 caused by the greater bioavailability of free oestrogens present in the endometrium of PCOS women (Leon *et al.* 2008) would lead to an impaired endometrial function (Lathi *et al.* 2002, Giudice 2006) that would further reduce chances of normal pregnancy.

Assuming that the bovine embryo resembles the human embryo behaviour in a high IGF1 microenvironment and that a concentration of ~1000 ng/ml is present in the oviducts and uterus of PCOS women, we propose that a substantial proportion of embryos developing in a PCOS environment may adapt (i.e. do not undergo degeneration) to the high IGF1 levels and develop to the blastocyst stage with normal cell allocation but increased apoptosis. However, a small, but significant, proportion of embryos will develop a hypertrophic ICM. If endometrial function is impaired, embryos with hypertrophic ICM are less likely to implant and undergo normal placentation. If this holds true, the risk of pregnancy loss would be greater in a small percentage of PCOS women with hyperinsulinaemia. A recent survey showed that the prevalence of recurrent miscarriage in PCOS (~10%) women is relatively low (Cocksedge *et al.* 2009). However, our model assumes that oocyte quality is not seriously compromised, which probably is not the case, as oocyte developmental competence was reduced in a PCOS-like bovine model of hyperinsulinaemia (Adamiak *et al.* 2005). Furthermore, we have accumulated evidence that *in vivo* oocyte developmental competence can be impaired by high concentrations of IGF1 (MA Velazquez, K-G Haderl, D Herrmann, WA Kues, S Ulbrich, HHD Meyer, B Rémy, J-F Beckers, H Sauerwein & H Niemann 2010, unpublished data).

In conclusion, we have shown that supraphysiological concentrations of IGF1 have detrimental effects on preimplantation embryo development primarily by increasing apoptosis and altering cell allocation towards the ICM. However, our results do not support the premise that increased apoptosis associated with high levels of IGF1 is mediated via downregulation of the IGF1R as previously found in preimplantation mouse embryos. At present, we have no explanation for the observed apoptosis induction without IGF1R downregulation in bovine embryos exposed to a high IGF1 microenvironment. Cellular signalling networks can be affected by IGF1 in bovine embryos and species differences in IGF signalling could be promising to unravel this phenomenon.

Materials and Methods

Collection and maturation of cumulus–oocyte complexes

Bovine ovaries from a local abattoir were transported to the laboratory at 25–30 °C in PBS (A0964; Applichem, Darmstadt, Germany), supplemented (enriched PBS) with 60 µg/ml penicillin G potassium (A1837, Applichem), 47 µg/ml streptomycin sulphate (A1852, Applichem), 36 µg/ml pyruvate (A3912, Applichem), 1.1 mg/ml α -D-glucose monohydrate (6780.1; Carl Roth GmbH, Karlsruhe, Germany) and 133 µg/ml calcium chloride dehydrate (21098; Fluka, Sigma–Aldrich). Ovaries were washed with 0.9% (w/v) sodium chloride (3957.2, Carl Roth GmbH) supplemented with 60 µg/ml penicillin G potassium and 1.3 µg/ml streptomycin sulphate. The surface of the ovaries was sliced in enriched PBS supplemented with 2.2 IU/ml heparin (24590; Serva, Heidelberg, Germany) and 0.1% (w/v) BSA fraction V (A1391, Applichem). Cumulus–oocyte complexes (COCs) were collected in tissue culture medium (TCM) air (TCM 199, with Earle's salts, L-glutamine and 25 mM HEPES, M2520; Sigma–Aldrich) supplemented with 22 µg/ml pyruvate, 50 µg/ml gentamicin sulphate (G3632, Sigma–Aldrich), 350 µg/ml sodium bicarbonate (NaHCO₃, 31437; Sigma–Aldrich) and 0.1% (w/v) BSA (fatty acid free, A7030; Sigma–Aldrich). Oocytes with at least three layers of compact cumulus cells and homogeneous granulated cytoplasm were selected (Racedo *et al.* 2008) and washed in groups of 15–20 in TCM culture medium (TCM air with a higher concentration of NaHCO₃ (2.2 mg/ml)). COCs were then placed into 100 µl drops of maturation medium (TCM culture supplemented with 10 IU/ml of equine chorionic gonadotrophin, 5 IU/ml of human chorionic gonadotrophin (Suigonan; Intervet, Unterschleißheim, Germany) and 100 µM cysteamine hydrochloride (M6500, Sigma–Aldrich)) and were matured (*in vitro* maturation, IVM) under a humidified atmosphere of 5% CO₂ in air at 39 °C for 24 h.

IVF

The medium for IVF was Fert-TALP medium (Parrish *et al.* 1988) with some modifications, such as omission of glucose, supplementation with 0.1 IU/ml heparin, 0.01 µg/ml phenol red (Merck, Darmstadt, Germany) and a higher amount of sodium pyruvate (28 µl/ml). A basic stock solution of Fert-TALP medium without BSA (6 mg/ml, A9647, Sigma–Aldrich), gentamicin sulphate (50 µg/ml), sodium pyruvate, hypotaurine (10 µM, H1384; Sigma–Aldrich), heparin and epinephrine (1 µM, E4250; Sigma–Aldrich) was prepared in advance. Matured COCs were washed with basic Fert-TALP medium supplemented with BSA, gentamicin sulphate and sodium pyruvate but lacking hypotaurine, heparin and epinephrine (HHE). The washed COCs were then placed in 100 µl drops of Fert-TALP medium used for washing but supplemented with HHE (IVF drops). Semen of one bull of proven fertility for IVF was thawed at 30 °C and layered on top of 750 µl of 90% (v/v) Percoll/Fert-TALP solution (P1644, Sigma–Aldrich) equilibrated at room temperature in a 1.5 Eppendorf tube. After centrifugation (400 g) for 16 min, the supernatant was aspirated and

the pellet resuspended with 750 µl of Fert-TALP without HHE. Centrifugation was applied again for 3 min followed by supernatant aspiration and pellet resuspension with Fert-TALP plus HHE. After a third 3 min centrifugation, the supernatant was aspirated leaving ~50 µl of sperm suspension. Sperm concentration was assessed and adjusted to 1×10^6 sperm/ml, corresponding to ~2 µl sperm suspension per IVF drop. COCs were co-incubated with sperm for 18 h under the same grouping and atmospheric conditions used for IVM.

In vitro culture

The modified SOF medium described by Holm *et al.* (1999) without tri-sodium citrate but supplemented with 4 mg/ml fatty acid-free BSA (A7030, Sigma–Aldrich) was used for *in vitro* culture (IVC). After IVF, presumptive zygotes were denuded by gentle vortexing. Zygotes were then washed three times in 80 µl drops of SOF medium followed by random allocation into 30 µl drops of SOF medium without IGF1 (control group) or supplemented with either 100 ng/ml IGF1 (physiological concentration) or 1000 ng/ml IGF1 (supraphysiological concentration). In each replicate, 35–56 zygotes were used per group and the number of drops and embryos per drop (5–8 zygotes) was kept equal among the groups. For the IGF1 treatments, a vial containing 50 µg of lyophilised recombinant human IGF1 (291-G1, R&D systems, Wiesbaden-Norderstadt, Germany) was rehydrated with 500 µl of 0.1% PBS/BSA according to the manufacturer's recommendations and stored at –20 °C in 10 µl aliquots (1000 ng). Culture drops containing IGF1 were prepared from two thawed aliquots diluted with SOF medium to the required concentrations of IGF1. Embryos were cultured in a humidified atmosphere containing 5% O₂, 5% CO₂ and 90% N₂ (Air products, Hattingen, Germany) at 39 °C. Drops used in each IVP cycle (i.e. IVM, IVF and IVC) were covered with silicone oil (35135, Silicone DC 200 fluid; Serva) and equilibrated for at least 3 h before use. Cleavage rate (embryos with four or more cells) and blastocyst formation were evaluated on days 3 and 8 after IVF (day 0) respectively. In each experimental group, cellular and molecular characteristics were evaluated only in day-8-expanded blastocysts obtained from 31 IVP cycles unless otherwise indicated.

Differential cell staining

Differential cell staining of blastocyst was carried out using a modified protocol described by Thouas *et al.* (2001). Briefly, zona-intact blastocysts were washed three times in 50 µl drops of 0.1% (w/v) polyvinylpyrrolidone/PBS and placed into 500 µl of 1% (v/v) Triton-X 100/PBS containing 100 µl/ml propidium iodide (PI) (P4170, Sigma–Aldrich) for 40 s. Embryos were then transferred into 500 µl of 100% ethanol containing 25 µl/ml bisbenzimidazole (Hoechst 33258, B 2883, Sigma–Aldrich) for 5 min. Blastocysts were mounted onto a glass microscope slide in an ~4 µl drop of glycerol and coverslipped. Cell counting was performed immediately after staining. Digital photographs of blastocysts were obtained with an epifluorescence microscope (Olympus BX60, Olympus Optical Co., Ltd, Tokyo, Japan) equipped with a digital camera (Olympus DP71) in a darkened room. Cell nuclei were counted using

the touch-count mode of the Olympus cell^P imaging software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Overlapping or out-of-focus nuclei in the digital photographs were identified by manual focus scanning of the samples under the microscope at the time of counting. Results are based on 11 IVP cycles.

Detection of apoptosis by TUNEL

Apoptotic nuclei were detected using an *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) based on the TUNEL principle using a previously described method (Zaraza *et al.* 2010). Briefly, zona-intact blastocysts were fixed in 4% (w/v) paraformaldehyde/PBS solution for 1 h followed by permeabilisation with Triton X-100 solution (0.5% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate/PBS) for 1 h at room temperature. Embryos were then incubated in 25 µl drops of TUNEL reaction mixture (10 µl of terminal deoxynucleotidyl transferase (TdT) and 90 µl of fluorescein-conjugated dUTP) for 1 h at 37 °C in the dark. Positive and negative controls were incubated with RNA-free DNase I solution (50 U/ml, D9905K; Epicentre Biotechnologies, Madison, WI, USA) at 37 °C for 1 h in the dark. After DNase incubation, positive controls were placed in the TUNEL reaction mixture and negative controls were incubated in the labelling solution in the absence of TdT. Thereafter, embryos were incubated in 50 µl drops of RNase A solution (50 µl/ml, R5500, Sigma–Aldrich) for 1 h at 37 °C in the dark followed by nuclei counterstaining in 50 µl drops of PI (50 µl/ml) for 15 min in the dark at room temperature. Blastocysts were then exposed to an increasing gradient (50, 75 and 100%) of Vectashield antifade mounting medium/PBS (v/v, H-1000; Vector Laboratories, Burlingame, CA, USA) at room temperature in the dark and mounted individually in 100% Vectashield. Slides were stored at 4 °C in the dark for up to 7 days before confocal laser-scanning microscopy (CLSM) was performed. The embryos were subjected to CLSM with a Zeiss LSM 510 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) using a Plan-Apochromat 20×/0.75 objective. FITC (excitation wavelength at 488 nm) and PI (excitation wavelength at 543 nm) were detected with an argon and helium laser respectively. Optical sections of 1.98 µm thickness were made at 4 µm intervals through the whole embryo and analysed with the LSM Image Browser software (Carl Zeiss MicroImaging GmbH). TCN and apoptotic nuclei were recorded. Nuclei were regarded as apoptotic only if they displayed both morphological (nuclear condensation with or without fragmentation) and biochemical (TUNEL positive) characteristics of apoptosis (Gjørret *et al.* 2003). Results are based on embryos obtained from eight IVP cycles.

Immunofluorescence

Zona-intact blastocysts from six IVP cycles were fixed and permeabilised as described in the TUNEL protocol. Following permeabilisation, embryos were blocked with 10% (v/v) new born calf serum/PBS (B15-001; PAA Laboratories GmbH, Pasching, Austria) for 1 h at room temperature followed by incubation overnight at 4 °C in the dark with the primary antibody diluted in 3% (w/v) BSA/PBS (A9647, Sigma–Aldrich).

The TP53 antibody (rabbit polyclonal, 9282, Cell Signalling Technology, Inc., Danvers, MA, USA) was diluted 1:100 (Favetta *et al.* 2004) and the IGF1R (rabbit polyclonal, PAI-14212; Dianova, Hamburg, Germany) 1:50. Embryos were then washed twice in Triton X-100 solution and incubated with the Alexa Fluor 488-conjugated secondary antibody (1:100 in 3% BSA/PBS; Invitrogen Ltd) for 2 h in the dark at room temperature. Embryos from the three groups were incubated in drops of equal volume obtained from the same antibody preparation. Later, embryos were treated with RNase, counterstained and mounted as described for the TUNEL protocol. Embryos not incubated with the primary antibody served as negative controls. Blastocysts were subjected to CLSM using the same settings as described in the TUNEL protocol. All embryos were scanned under the same confocal settings. Optical sections were analysed with LSM Image Browser software. After TCN counting, the fluorescence intensity of individual embryos was assessed in one central optical section displaying both the ICM and the TE by marking and extracting six small areas of equal size. Extracted images were saved in tagged image file format (TIFF) and processed according to the method developed by Tolivia *et al.* (2006) and validated in bovine embryos (Favetta *et al.* 2007). Briefly, green channel images (representing Alexa Fluor 488 fluorescence) were converted to 'greyscale' and then inverted with Adobe Photoshop CS2 (Adobe Systems Inc.) so that grey and black pixels represented the areas of Alexa Fluor 488 immunofluorescence on a white background. Inverted images were saved as a new TIFF file and opened in the Scion Image program (4.0.3.2, Scion Corporation, Frederick, MD, USA) to obtain the mean density of the chromogen signal strength (SS). The SS was normalised to the maximum SS obtained in each image in order to produce a relative SS (Tolivia *et al.* 2006). The relative SS in individual embryos was the mean value of the small sections extracted per embryo (i.e. six for the whole embryo and three per cell compartment (ICM and TE)).

mRNA isolation

Blastocysts from six IVP cycles were individually placed into 0.6 ml siliconised Eppendorf tubes with ~4 µl of 0.1% (w/v) polyvinyl alcohol/PBS solution and stored at –80 °C until mRNA extraction. Poly (A)⁺ RNA was isolated using a Dynabeads mRNA DIRECT micro kit (610.21, Invitrogen Dynal AS) with some modifications to the manufacturer's instructions as previously described (Kues *et al.* 2008, Niemann *et al.* 2010). Briefly, 40 µl of lysis-binding buffer (100 mM Tris–HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulphate (LiDS), 5 mM dithiothreitol) were pipetted into each sample tube, followed by addition of 1 µg rabbit globin mRNA (Bethesda Research Laboratories, Gaithersburg, MD, USA) as external standard. After a short centrifugation, samples were incubated at room temperature for 10 min. In all, 5 µl of prewashed Dynabeads Oligo (dT)₂₅ were then added into each lysate and placed on a shaker at room temperature for 15 min to allow binding of the poly (A)⁺ RNA to the Dynabeads. The beads were then washed once with 40 µl buffer A (10 mM Tris–HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) and three times with 40 µl buffer B (10 mM Tris–HCl, pH 8.0, 0.15 M

LiCl, 1 mM EDTA) at room temperature. After washing, beads were resuspended with 11 µl ice-cold sterile water and the poly (A)⁺ RNAs were eluted from the beads by incubation at 65 °C for 2.5 min followed by flash cooling on ice. The 11 µl elution aliquots were used immediately for RT.

RT

Poly (A)⁺ RNA from single blastocysts was reverse transcribed into first strand cDNA in a 0.2 ml reaction tube containing 20 µl reaction mixture. The RT reaction mixture consisted of 2 µl (1×, 20 mM Tris-HCl, pH 8.4, 50 mM KCl) of 10× RT buffer (Invitrogen), 2 µl (5 mM) of magnesium chloride (Invitrogen, Karlsruhe, Germany), 2 µl (1 mM) of dNTP solution (Amersham Biosciences Europe), 1 µl (2.5 µM) of random hexamer primers (N808-0017, GeneAmp RNA PCR kit; Applied Biosystems, Darmstadt, Germany), 1 µl (20 U) of RNase inhibitor (N808-0119, Applied Biosystems), 1 µl (50 U) of murine leukaemia virus reverse transcriptase (N808-0018, Applied Biosystems) and the 11 µl of mRNA preparation. Tubes with reaction mixture containing sterile water instead of mRNA preparation were used as controls for contamination. A tube with reaction mixture and 2 µl (1 pg) of rabbit globin mRNA and 9 µl of sterile water was prepared to produce a pool of cDNA globin for quantification of globin expression used for data normalisation (see below). The RT reaction was carried out in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) and consisted of 10 min at 25 °C for primer annealing, 1 h at 42 °C for primer extension and 5 min at 95 °C to terminate the reaction followed by cooling down at 8 °C and storage at –25 °C until real-time PCR analysis.

Quantitative real-time PCR

For real-time PCR, wells from 96-well optical reaction plates were loaded with 20 µl PCR reaction mixture containing 10 µl power SYBR green PCR master mix (4367659, Applied Biosystems), 0.8 µl (5 µM) each of the forward and reverse primers of the respective genes of interest (Table 3), 2 µl cDNA (0.2 blastocyst equivalents) and 6.4 µl sterile water. Amplification was carried out in an ABI 7500 Fast Real-Time System (Applied Biosystems). The PCR reaction started with 10 min at

95 °C for activation of the Taq DNA Polymerase, followed by 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Post PCR, dissociation melting curve analyses were carried out to determine the specificity of the PCR-amplified products. Raw gene expression data for target genes and globin were obtained with the Sequence Detection Software 1.3.1 (Applied Biosystems) based on standard curve dilution series (1:5) of cDNA from 60 blastocysts and pooled globin respectively (Niemann *et al.* 2010). Data were then transferred to Microsoft excel and the relative mRNA abundance was calculated by dividing the target gene expression value by the amount of globin mRNA expressed in each sample (globin added as external control during RNA extraction). To normalise data to embryo cell number, the relative abundance of each transcript in individual embryos was divided by the mean TCN (Table 2) and multiplied by 100 (Block *et al.* 2008). For each group, results are based on 12 replicates per gene.

Statistical analysis

Data were analysed with SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA). Differences between the groups were tested by χ^2 test and ANOVA with the Fisher's least significant difference method as *post hoc* test. Associations between variables were tested by regression analysis. Percentage values analysed as continuous data were arcsine transformed before analysis. Continuous data were transformed if they did not meet the assumption of normal distribution or homogeneity of variance. If no improvement was achieved after transformation, non-parametric equivalents were used (e.g. the Kruskal-Wallis test). When a *P* value with a tendency towards significance (<0.08) was detected with ANOVA or the Kruskal-Wallis test, a two-group comparison test was carried out (e.g. Student's *t*-test) to clarify the differences between treatments. Fluorescence intensity within the groups (i.e. ICM versus TE) was analysed by *t*-test or the Mann-Whitney test as appropriate. Since data were analysed by parametric and non-parametric tests, results are presented as mean $\pm$ S.E.M. and median (Q1–Q3) unless otherwise indicated. *P* ≤ 0.05 was considered to be statistically significant.

Table 3 Primers used for real-time PCR.

Genes	Primer sequences and positions		Annealing temperature (°C)	Fragment size (bp)	Accession number
<i>IGF1R</i>	5' (1068–1090)	CCTCATCAGCTTCACCGTCTACT	60	72	XM_606794.3
	3' (1139–1121)	GCGTCCTGCCCGTCATACT			
<i>IGFBP3</i>	5' (731–752)	AACTTCTCCTCTGAGTCCAAGC	60	210	NM_174556.1
	3' (941–921)	CGTACTTATCCACACACCAGC			
<i>TP53</i>	5' (720–739)	TTTACGCGCGGAGTATTGG	60	57	NM_174201.2
	3' (776–756)	GGCACCACCACACTGTGTCTA			
<i>AKT1</i>	5' (368–385)	GCTCACCCGCGGAGAACT	60	108	AY781100.1
	3' (457–475)	CTTTGCCAGCAGCTTCAG			
<i>SLC2A1</i>	5' (894–914)	CAGGAGATGAAGGAGGAGAGC	60	258	NM_174602.2
	3' (1131–1151)	CACAAATAGCGACACGACAGT			
<i>SLC2A3</i>	5' (127–149)	GTTGCTACCATAGGCTCTTTCCA	60	65	AY033938
	3' (173–192)	GATCGCCTCAGGAGCATTGA			
<i>SLC2A8</i>	5' (1441–1461)	GCATCTTCGGTGTCTCTTTTCA	60	80	AY208940
	3' (1501–1521)	CAAAATGGGCTGTGATTGCT			

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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