***In vivo* oocyte developmental competence is reduced in lean but not in obese superovulated dairy cows after intraovarian administration of IGF1**

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Abstract

The present study investigated the role of IGF1 in lactating lean and non-lactating obese dairy cows by injecting 1 µg IGF1 into the ovaries prior to superovulation. This amount of IGF1 has been linked with pregnancy loss in women with the polycystic ovary syndrome (PCOS) and was associated with impaired bovine oocyte competence *in vitro*. Transcript abundance and protein expression of selected genes involved in apoptosis, glucose metabolism, and the IGF system were analyzed. Plasma concentrations of IGF1 and leptin, and IGF1 in uterine luminal fluid (ULF), were also measured. IGF1 treatment decreased embryo viability in lean cows to the levels observed in obese cows. Obese cows were not affected by IGF1 treatment and showed elevated levels of IGF1 (in both plasma and ULF) and leptin. Blastocysts from lean cows treated with IGF1 showed a higher abundance of *SLC2A1* and *IGFBP3* transcripts. IGF1 treatment reduced protein expression of tumor protein 53 in blastocysts of lean cows, whereas the opposite was observed in obese cows. IGF1 in plasma and ULF was correlated only in the control groups. Blastocyst transcript abundance of IGF1 receptor and *IGFBP3* correlated positively with IGF1 concentrations in both plasma and ULF in lean cows. The detrimental microenvironment created by IGF1 injection in lean cows and the lack of effect in obese cows resemble to a certain extent the situation observed in PCOS patients, where IGF1 bioavailability is increased in normal-weight women but reduced in obese women, suggesting that this bovine model could be useful for studying IGF1 involvement in PCOS.

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Introduction

The insulin-like growth factor (IGF) system is critical for ovarian function (Kwintkiewicz & Giudice 2009) and is an important signaling mechanism disrupted by hyperinsulinemia in women with the polycystic ovary syndrome (PCOS; Essah *et al.* 2004). High insulin concentrations reduce synthesis of IGF binding proteins such as IGFBP1, which in turn enhances the bioactivity of IGF1 (Thierry van Dessel *et al.* 1999, Wang & Chard 1999). *In vivo* and *in vitro* rodent models demonstrated that exposing embryos to supraphysiological concentrations of IGF1 can result in abnormal preimplantation embryo development (Katagiri *et al.* 1996, 1997). This led to the hypothesis of a link between high levels of IGF1 and early pregnancy loss in PCOS women

(Chi *et al.* 2000, Eng *et al.* 2007, Pinto *et al.* 2002). The murine PCOS model was used to test the effects of supraphysiological concentrations of IGF1 during *in vitro* preimplantation embryo development (Chi *et al.* 2000, Pinto *et al.* 2002, Moley *et al.* 2005, Eng *et al.* 2007) but studies investigating *in vivo* oocyte developmental competence are lacking. *In vitro* oocyte developmental competence was reduced after nutrient-induced maternal hyperinsulinemia of bovine oocyte donors, similar to the insulin values observed in PCOS patients (Adamiak *et al.* 2005). The bovine female has been proposed as an *in vivo* bioassay for the generation of conceptual models relevant to ovarian function in women (Adams & Pierson 1995) and has played a pivotal role in the development of an *in vivo* culture system

for human embryos (Blockeel *et al.* 2009). Recently, the superovulated bovine female was proposed as an alternative animal model to investigate the endocrine-related pathologies associated with IGF1 such as PCOS (Velazquez *et al.* 2009). In fact, the nymphomaniac cow has been considered as the only naturally occurring animal model for PCOS (Abbott *et al.* 2006).

The goal of the present study was to evaluate the effects of priming ovaries with intraovarian injections of IGF1 upon the superovulatory response of lactating lean and non-lactating obese dairy cows, as the bioavailability of IGF1 seems to be increased in normal-weight PCOS women but reduced in obese PCOS patients (Silfen *et al.* 2003, Premoli *et al.* 2005, Pasquali & Gambineri 2008). IGF1 (1 µg) was injected into the ovarian stroma, similar to the concentrations suggested to be present in PCOS women experiencing early pregnancy loss (i.e. 950–1500 ng/ml; Chi *et al.* 2000, Eng *et al.* 2007, Pinto *et al.* 2002) and associated with detrimental effects on bovine oocyte competence *in vitro* (Thomas *et al.* 2007). We used the bovine model established in our laboratory in which we have shown that an intraovarian injection of IGF1 affects mRNA expression of oocytes recovered by ovum pick-up, indicating that the IGF1 injected indeed reaches the oocyte in the ovarian follicle (Oropeza *et al.* 2004, Zaraza *et al.* 2010). It was intended to mimic the intraovarian milieu to which oocytes are exposed during the final stages of ovarian follicular development in women with PCOS. Embryo yields were determined and the resultant blastocysts were analyzed for specific characteristics related to the IGF pathway, including protein expression of the IGF1 receptor (IGF1R) and tumor protein 53 (TP53 gene), and transcript abundance of selected genes involved in apoptosis, glucose metabolism, and the IGF system. Furthermore, concentrations of IGF1 in plasma and non-diluted uterine luminal fluid (ULF) were measured.

Results

Superovulatory response

In the first trial, lean cows treated with IGF1 had a significantly reduced rate of viable embryos and an increased rate of degenerated embryos compared with the control group (Fig. 1). None of the superovulatory parameters was affected by IGF1 treatment in obese cows in both trials (Fig. 1 and Table 1). Parameters of embryo viability were similar between IGF1-treated lean cows and obese cows (Fig. 1). Lean control cows had a higher viability rate compared with obese cows treated with IGF1 (Fig. 1). The rate of degenerated embryos was lower in the control group of lean cows compared with the rest of the groups (Fig. 1). Most of the numerical parameters (e.g. number of viable embryos) of superovulatory response were higher in lean control cows

Figure 1 Effect of intraovarian application of 1 µg IGF1 on selected parameters of superovulatory response expressed as proportions in lactating lean and non-lactating obese cows. Within trials, bars with different superscripts indicate a significant difference ($P \leq 0.05$).

compared with obese cows (Table 1). Bovine superovulatory outcome can be compromised by lactation (Leroy *et al.* 2005), but the superovulatory response in control animals of this study was strongly related to the energy status of donors (reflected in their body condition score (BCS)) and not to lactation status *per se*, as lactating cows (lean animals) displayed a high production of viable embryos compared to non-lactating cows (obese animals).

mRNA transcript expression

Blastocysts from lean cows treated with IGF1 showed significantly increased expression of *IGFBP3* and *SLC2A1* compared with their control counterparts (Fig. 2). Transcript abundance of embryos from obese cows was not affected by IGF1 treatment. Blastocysts from lean cows treated with IGF1 expressed more *IGFBP3* transcripts than blastocysts collected from IGF1-treated obese cows (Fig. 2). Blastocysts from lean cows treated with IGF1 also expressed more *SLC2A1* transcripts than blastocysts collected from control obese cows (Fig. 2).

IGF1R immunofluorescence

IGF1R was localized to the cell membrane and the cytoplasm in both the inner cell mass (ICM) and trophectoderm (TE) in all embryos analyzed (Fig. 3). There were no significant differences in relative signal

Table 1 Effects of intraovarian application of 1 µg IGF1 on the superovulatory response of lean lactating and obese non-lactating cows (summary of both trials).

Parameter ^a	Lean cows		P	Differences between groups P	Obese cows		P
	Control (23) ^b	IGF1 (24) ^b			Control (15) ^b	IGF1 (21) ^b	
Corpora lutea (CL)	14.9 ± 1.5 15.0 (8.5–19.2)	11.3 ± 1.6 9.5 (5.5–17.0)	0.128	A=0.004; B=0.309; C=0.008; D=0.183	8.2 ± 1.1 8.0 (4.2–11.0)	9.1 ± 1.2 10.0 (4.7–12.2)	0.613
Ova + embryos	13.2 ± 1.7 13.0 (5.2–19.2)	9.0 ± 1.8 6.0 (1.0–15.5)	0.111	A=0.007; B=0.731; C=0.010; D=0.563	6.1 ± 1.3 6.0 (1.2–10.5)	7.0 ± 1.3 5.0 (2.7–11.0)	0.665
Embryos	11.4 ± 1.7 10.0 (4.0–17.0)	8.2 ± 1.7 5.0 (1.0–15.0)	0.116	A=0.007; B=0.458; C=0.005; D=0.311	4.7 ± 1.2 2.0 (1.0–7.7)	5.3 ± 1.0 4.0 (2.0–9.2)	0.647
Viable embryos	9.9 ± 1.5 10.0 (4.0–14.7)	6.0 ± 1.4 3.5 (0.0–11.0)	0.033	A=0.006; B=0.262; C=0.004; D=0.412	3.6 ± 1.0 1.0 (0.0–6.0)	4.0 ± 0.8 3.0 (0.7–6.5)	0.603
Quality 1 embryos	7.3 ± 1.1 6.0 (3.2–12.7)	4.6 ± 1.1 3.5 (0.0–7.5)	0.041	A=0.009; B=0.254; C=0.004; D=0.286	3.0 ± 0.8 1.0 (0.0–5.7)	3.0 ± 0.7 2.0 (0.0–6.0)	0.936
Quality 2 embryos	2.6 ± 0.6 1.0 (0.0–4.0)	1.3 ± 0.4 0.0 (0.0–2.5)	0.153	A=0.030; B=0.820; C=0.097; D=0.277	0.6 ± 0.4 0.0 (0.0–0.0)	1.0 ± 0.3 0.0 (0.0–0.2)	0.318
Quality 3 embryos	0.4 ± 0.2 0.0 (0.0–0.0)	0.1 ± 0.1 0.0 (0.0–0.0)	0.756	A=0.718; B=0.757; C=0.991; D=0.930	0.6 ± 0.6 0.0 (0.0–0.0)	0.1 ± 0.1 0.0 (0.0–0.0)	0.697
Morulae	3.8 ± 0.8 3.0 (0.0–7.7)	3.1 ± 0.8 1.0 (0.0–5.5)	0.406	A=0.075; B=0.624; C=0.126; D=0.394	1.5 ± 0.5 0.0 (0.0–3.2)	1.8 ± 0.5 1.0 (0.0–3.2)	0.585
Blastocysts	6.5 ± 1.3 5.0 (1.0–11.2)	3.0 ± 0.8 0.5 (0.0–5.5)	0.040	A=0.017; B=0.900; C=0.027; D=0.654	2.1 ± 0.9 0.0 (0.0–3.5)	2.4 ± 0.6 2.0 (0.0–4.0)	0.480
Degenerated embryos	1.0 ± 0.4 0.0 (0.0–1.0)	2.0 ± 0.6 0.5 (0.0–3.0)	0.388	A=0.600; B=0.592; C=0.742; D=0.729	1.0 ± 0.3 1.0 (0.0–2.0)	1.0 ± 0.3 0.0 (0.0–2.0)	0.860
Unfertilized ova	1.7 ± 0.6 0.0 (0.0–1.7)	0.7 ± 0.4 0.0 (0.0–1.0)	0.382	A=0.753; B=0.368; C=0.991; D=0.183	1.4 ± 0.4 1.0 (0.0–2.7)	1.6 ± 0.6 0.0 (0.0–2.0)	0.664

A=lean control (L-C) versus obese control (O-C), B=lean IGF1 (L-IGF1) versus obese IGF1 (O-IGF1), C=L-C versus O-IGF1, D=L-IGF1 versus O-C.
^aReported as mean ± s.e.m. and median (first quartile to third quartile). ^bAnalysis was carried out only in cows that responded to superovulation (i.e. two or more CL at the time of embryo recovery).

strength (RSS) values between the ICM and TE in blastocysts from lean cows. In the obese group, only blastocysts from control cows showed less IGF1R in the ICM (Fig. 4). IGF1 treatment did not affect blastocyst IGF1R expression in any of the groups. Embryos from lean cows had a higher protein expression of the IGF1R than those from obese control cows (Fig. 4). Blastocysts collected from obese cows treated with IGF1 and lean control animals did not show significant differences. However, embryos from IGF1-treated lean cows displayed a higher IGF1R expression compared with embryos from obese cows treated with IGF1 (Fig. 4).

TP53 immunofluorescence

All embryos showed cell membrane and cytoplasmic localization of TP53 in both the ICM and the TE (Fig. 3). No differences in RSS values were detected between the ICM and TE in any of the groups. In the lean group, blastocysts from cows treated with IGF1 showed a reduced expression of TP53 protein compared with controls. In contrast, in obese animals, blastocysts from IGF1-treated cows displayed an increased TP53 expression compared with controls (Fig. 4). Embryos from lean control cows had more TP53 than embryos from obese cows (Fig. 4). Blastocysts collected from lean cows treated with IGF1 expressed more TP53 than those from obese control cows, but showed a lower TP53 expression than blastocysts from obese IGF1-treated cows (Fig. 4).

Embryo cell number, concentrations of IGF1 in plasma and ULF, and plasma concentrations of leptin

Blastocysts from lean control cows had more total cells than embryos from the other groups (Table 2). Intraovarian application of IGF1 did not affect plasma or ULF IGF1 concentrations in any of the groups. Obese cows showed higher concentrations of IGF1 in plasma compared with lean cows. The same was observed for IGF1 concentrations in ULF but only in the control groups (Table 2). Plasma and ULF IGF1 concentrations correlated only in the control groups (lean cows: $r=0.516$, $r^2=0.23\%$, $P=0.014$; obese cows: $r=0.712$, $r^2=0.54\%$, $P=0.002$). IGF1 concentrations in plasma and ULF were correlated with the expression of IGF1R (plasma: $r=0.502$, $r^2=0.20\%$, $P=0.034$; ULF: $r=0.554$, $r^2=0.26\%$, $P=0.017$) and IGFBP3 (plasma: $r=0.476$, $r^2=0.17\%$, $P=0.046$; ULF: $r=0.625$, $r^2=0.35\%$, $P=0.006$) in blastocysts collected from lean control cows. For both genes, the correlation was stronger with IGF1 concentrations in ULF. Leptin concentrations in plasma were not affected by IGF1 treatment in any of the groups (Table 3). However, the obese cows displayed higher plasma concentrations of leptin than did the lean cows (Table 3).

Discussion

The main finding of the present study is the negative effect of intraovarian application of IGF1 on oocyte developmental competence observed exclusively in lean

Figure 2 Relative transcript abundance (mean $\pm$ S.E.M.) of developmentally important genes in blastocysts collected from control and IGF1-treated lactating lean and non-lactating obese superovulated cows. Bars with different superscripts within each gene transcript indicate a significant difference ($P \leq 0.05$). Each analysis was replicated 7–18 times.

lactating cows, which could at least be partially explained by interactions of IGF1 with other hormones of reproductive importance, such as leptin. As previously reported (Ehrhardt *et al.* 2000, Adamiak *et al.* 2005), obese cows in the present study had higher leptin concentrations in blood than their lean counterparts. A positive correlation between bovine leptin levels in blood and follicular fluid has been documented (Dayi *et al.* 2005). The hormonal differences between lean and obese cows were expected, as it is well documented that IGF1 and leptin are closely associated with BCS (Ehrhardt *et al.* 2000, Velazquez *et al.* 2008). Our hypothesis was that an oocyte in a microenvironment with no deficits of IGF1 (lean cows out of the phase of negative energy balance) will be negatively affected by inducing a short high IGF1 microenvironment similar to the one present in PCOS women. Therefore, a marked detrimental effect of IGF1 treatment was expected in

obese cows that should have higher concentrations of IGF1. However, this was not the case. *In vitro* experiments have demonstrated that high concentrations of leptin can suppress the stimulatory effects of IGF1 upon steroidogenesis in ovarian follicular cells from rats (Zachow & Magoffin 1997), humans (Agarwal *et al.* 1999, Huang *et al.* 2005, Karamouti *et al.* 2008), and cattle (Spicer *et al.* 2000). Based on these findings, we choose to analyze leptin levels as a starting point to elucidate why the IGF1 treatment did not affect the superovulatory response in obese cows. The effects of exogenous IGF1 on ovarian follicles were probably partially neutralized by leptin in obese cows. Nevertheless, this hypothesis requires further study, including the underlying mechanism of the neutralizing effect of leptin on IGF1 action. This could involve reduced expression of the IGF1R, as ovarian follicular gene expression of the IGF1R can be decreased after *in vivo* infusion of pharmacological doses of leptin in ruminants (Muñoz-Gutiérrez *et al.* 2005). In the present study, blastocysts from obese control cows displayed reduced protein expression of the IGF1R compared with lean control cows.

To our knowledge, this is the first report providing *in vivo* evidence that IGF1 can upregulate its receptor

Figure 3 Confocal images showing immunolocalization of IGF1R and TP53 (green channel) in *in vivo* blastocysts from superovulated bovine donors. Both TP53 ($n = 75$ blastocysts) and IGF1R ($n = 53$ blastocysts) were localized in the cell membrane and the cytoplasm in both the inner cell mass and trophoblast in all embryos analyzed. Nuclei were counterstained with propidium iodide (PI, red channel). Merged images (green and red channel) showed no nuclear localization of both proteins.

Figure 4 Differences ($P \leq 0.05$) in relative signal strength (RSS) values (mean $\pm$ S.E.M.) of IGF1R and TP53 in blastocysts collected from control and IGF1-treated lactating lean and non-lactating obese superovulated cows. Bars with different small superscripts (a, b, c, and d) indicate significant differences between the inner cell mass (ICM) and trophoblast (TE) within groups (control or IGF1 (ICM versus TE)), and differences between groups (control versus IGF1) within categories (lean or obese cows) in the same cell compartment (ICM versus ICM; TE versus TE). Small superscripts also indicate differences in the same cell compartment (ICM versus ICM; TE versus TE) between lean and obese cows. Significant differences in total RSS values are indicated by capital superscripts (A, B, C, and D). *Significance obtained with Mann-Whitney U test (B versus C).

and one of its binding proteins in bovine preimplantation embryos. The coefficients of determination were relatively low, but not unexpected as transcript expression of these genes is also regulated by steroids, gonadotropins and other growth factors (Sepp-Lorenzino 1998, Firth & Baxter 2002). However, this relationship was only detected in lean cows not treated with IGF1. These cows also showed the highest yield of viable embryos and lowest proportion of degenerated embryos. This observation implies that subtle disruptions in the delicate balance of components in the ULF can be sufficient to interfere with preimplantation embryo development. An in-depth study of this relationship (i.e. correlation between embryo gene expression and hormonal concentrations in blood and ULF=optimal embryo development) could provide clues to identify the optimal uterine microenvironment for preimplantation embryo development. Our data also showed that, after intraovarian IGF1 application, the correlation between

IGF1 concentrations in plasma and ULF was lost. The reason for this is unknown, but it is possible that some of the injected IGF1 could have reached the oviducts and uterus via counter-current transfer (Einer-Jensen & Hunter 2005) and altered the transport of IGF1 across oviductal and uterine tissues, probably by altering vascular permeability (Nasu *et al.* 2006). Accordingly, as few as seven small IGF1-loaded beads placed into the uterine lumen of mice were capable of inducing changes in endometrial vascular permeability (Paria *et al.* 2001). Transfer from the ovary to oviducts and uterus could have occurred within seconds or minutes (Einer-Jensen & Hunter 2005) after IGF1 administration and was probably exacerbated in our model, as blood flow in reproductive tract is dramatically increased by superovulation (Honnens *et al.* 2009).

The present study confirmed the known adverse effects of obesity upon mammalian reproduction (Brewer & Balen 2010) and revealed that intraovarian application of high amounts of IGF1 in lean cows can decrease embryo viability to the levels observed in obese cows. We recently reported that intraovarian administration of 6 μ g IGF1 reduced blastocyst yields of cycling young heifers subjected to ovum pick-up and IVF compared with mock-treated control animals (Zaraza *et al.* 2010). In this model, the heifers were still growing and endogenous concentrations of IGF1 were probably higher than in their adult counterparts, with no deficit of IGF1, unless they are under a catabolic disease state (Velazquez *et al.* 2008). Our results also agree with previous studies carried out in women (Wang *et al.* 2006) and heifers (Nicholas *et al.* 2005, Siddiqui *et al.* 2009), where oocytes obtained from follicles with high IGF1 bioavailability (i.e. more free IGF1 or less IGFBPs) displayed impaired developmental competence *in vitro*. *In vitro* studies have shown that exposing bovine follicles to a high IGF1 microenvironment leads to reduced oocyte size and a decreased number of granulosa cells (McCaffery *et al.* 2000, Thomas *et al.* 2007). A recent *in vivo* experiment showed that intrafollicular injection of 200 μ g IGF1 in the second largest ovarian follicle decreased the growth rate and maximum diameter of the largest follicle (Shahiduzzaman *et al.* 2010). All these features are known to have negative consequences for meiotic maturation, fertilization, and development to the blastocyst stage (Fair *et al.* 1995, Arlotto *et al.* 1996, Otoi *et al.* 1997). Results of the present investigation also indicate that oocyte competence can be compromised by a relatively short exposure to high levels of IGF1. Similarly, short-term treatment (9 h) with physiological concentrations of IGF1 (100 ng/ml) was enough to block heat shock-induced apoptosis in preimplantation bovine embryos (Jousan *et al.* 2008). Experiments in humans and ruminants revealed that unbound IGF1 has a half-life of ~ 12 min, but it can be prolonged up to several hours when linked to binding proteins (Guler *et al.* 1989, Basset *et al.* 1990). Accordingly, IGFBPs can

Table 2 Embryo cell number and concentrations (ng/ml) of IGF1 in plasma and uterine luminal fluid (ULF) in superovulated lean lactating and obese non-lactating cows after intraovarian application of 1 µg IGF1.

	Lean cows		Differences between groups		Obese cows	
	Control	IGF1	P	P	Control	IGF1
First trial ^a						
IGF1-plasma	(17) ^b 33.3 ± 3.6 30.0 (26.5–40.9)	(17) ^b 34.2 ± 2.7 33.6 (27.0–37.3)	0.858	A = 0.001; B = 0.002; C = 0.003; D = 0.001	(6) ^b 70.8 ± 11.5 64.5 (46.6–101.3)	(9) ^b 59.1 ± 8.2 71.0 (39.2–77.7)
IGF1-ULF	3.2 ± 0.6 2.2 (1.1–4.2)	2.9 ± 0.6 2.2 (1.2–3.7)	0.960	A = 0.027; B = 0.003; C = 0.007; D = 0.020	7.8 ± 2.8 5.4 (2.0–14.3)	8.2 ± 2.3 6.5 (5.0–8.5)
Second trial ^a	(5) ^c 43.9 ± 9.0 39.9 (32.8–60.7)	(6) ^c 33.1 ± 4.4 35.9 (26.8–41.0)	0.288	A = 0.230; B = 0.003; C = 0.147; D = 0.003	(8) ^c 54.9 ± 3.9 56.4 (47.5–63.0)	(12) ^c 57.2 ± 4.2 56.7 (47.6–67.9)
IGF1-plasma	4.4 ± 1.1 4.9 (1.8–6.6)	3.7 ± 1.4 2.7 (1.6–4.6)	0.741	A = 0.323; B = 0.523; C = 0.198; D = 0.647	4.5 ± 0.8 3.7 (2.8–6.3)	2.9 ± 0.5 2.9 (1.4–3.9)
IGF1-ULF	186.9 ± 4.3 187.0 (168.2–205.5)	164.7 ± 6.2 163.0 (146.0–190.0)	0.004	A = 0.001; B = 0.725; C = 0.003; D = 0.438	157.0 ± 6.6 159.5 (133.0–168.0)	161.2 ± 7.5 152.0 (134.5–179.5)
Cell number	(22)	(23)			(14)	(21)
Both trials ^a						
IGF1-plasma	35.7 ± 3.4 34.8 (27.6–41.1)	33.9 ± 2.3 33.8 (26.9–40.0)	0.659	A = 0.001; B = 0.001; C = 0.001; D = 0.001	61.7 ± 5.6 56.4 (46.6–70.0)	58.0 ± 4.1 56.8 (43.5–74.8)
IGF1-ULF	3.4 ± 0.5 2.3 (1.3–4.9)	3.1 ± 0.5 2.2 (1.2–3.8)	0.728	A = 0.042; B = 0.066; C = 0.176; D = 0.021	5.9 ± 1.3 4.5 (2.7–7.9)	5.2 ± 1.1 4.0 (2.1–6.6)

A = lean control (L-C) versus obese control (O-C), B = lean IGF1 (L-IGF1) versus obese IGF1 (O-IGF1), C = L-C versus O-IGF1, D = L-IGF1 versus O-C.

^aReported as mean ± S.E.M. and median (first quartile to third quartile). ^bCows used to collect embryos for gene expression analysis. ^cCows used to collect embryos for immunostaining.

retain IGF1 in the apical region of bovine endothelial cells, controlling localized delivery of IGF1 (Paye & Forsten-Williams 2006). In the present study, the milieu of the oocyte affected not only development to blastocysts but also the molecular and cellular parameters measured at the blastocyst stage. Similar long-term effects have been previously reported. For instance, a short (30 min) exposure of pig oocytes to bovine oviductal fluid induced changes in the cell number and gene expression at the blastocyst stages (Lloyd *et al.* 2009).

Whether the raised expression of *SLC2A1* (formerly known as *GLUT1*) and *IGFBP3* detected in blastocysts from lean cows treated with IGF1 is indicative of impaired embryo quality is unknown at present. Expression of *SLC2A1* was not different between *in vivo*- and *in vitro*-produced embryos, even though a significant reduction in pregnancy rates was observed with *in vitro*-produced embryos (Lazzari *et al.* 2002). Bertolini *et al.* (2002) found that *in vivo*-derived embryos showed an increased transcript abundance of *SLC2A1* compared with embryos produced *in vitro*. In contrast, Rho *et al.* (2007) reported higher expression of *SLC2A1* in *in vitro*-derived embryos compared with their *in vivo* counterparts and suggested that this was an adaptive response due to altered metabolism. However, this adaptive response might not necessarily represent a decline in quality as shown by the upregulation of *SLC2A1* in blastocysts derived from a defined *in vitro* medium capable of having similar calving rates as those from *in vivo* embryos (Lim *et al.* 2007). The available information on *IGFBP3* indicates that *in vivo*-derived embryos expressed more *IGFBP3* transcripts than *in vitro*-produced embryos (Sawai 2009). TP53 expression plays a role in cell cycle arrest and apoptosis in tumor cells (Rodier *et al.* 2007), and activation of TP53 has been proposed to be indicative of embryo demise (Keim *et al.* 2001, Matwee *et al.* 2001). However, recent information suggests that TP53 is critical for successful development, as the primordial function of its ancestor gene was to ensure fecundity and production of normal offspring, and its function as tumor suppressor came later in evolution (Hu 2009). For instance, expression of TP53 is essential for female fertility (Hu *et al.* 2007) and active TP53-dependent cell death signaling is required to suppress erroneous replication of damaged DNA during the preimplantation period (Toyoshima 2009) and to avoid abnormalities during fetal development (Torchinsky & Toder 2010). In fact, normal cellular differentiation during murine embryogenesis has been associated with increased expression of TP53 (Schmid *et al.* 1991). From this perspective, the low levels of TP53 protein in blastocyst from lean cows treated with IGF1 could indicate reduced embryo quality, whereas the opposite could be implied in obese cows, as embryos from IGF1-treated cows in this group displayed higher expression of TP53.

Table 3 Concentrations (ng/ml) of leptin in plasma of superovulated lean lactating and obese non-lactating cows after intraovarian application of 1 µg IGF1.

	Lean cows		<i>P</i>	Differences between groups <i>P</i>	Obese cows		<i>P</i>
	Control	IGF1			Control	IGF1	
First trial ^a	(17) ^b	(17) ^b			(6) ^b	(9) ^b	
Leptin-plasma	5.1 ± 0.4 4.6 (4.2–6.2)	5.1 ± 0.4 4.7 (3.9–5.9)	0.977	A=0.001; B=0.001; C=0.001; D=0.001	8.5 ± 0.8 9.1 (6.5–9.8)	9.5 ± 0.9 10.3 (7.4–10.7)	0.461
Second trial ^a	(5) ^c	(6) ^c			(8) ^c	(12) ^c	
Leptin-plasma	4.7 ± 0.5 4.4 (3.7–5.7)	4.3 ± 0.1 4.2 (4.1–4.5)	0.931	A=0.002; B=0.001; C=0.030; D=0.001	8.0 ± 0.5 8.1 (6.6–9.3)	8.0 ± 0.8 7.3 (5.9–8.7)	0.999
Both trials ^a	(22)	(23)			(14)	(21)	
Leptin-plasma	5.0 ± 0.3 4.5 (4.1–6.2)	4.9 ± 0.3 4.5 (4.0–5.5)	0.829	A=0.001; B=0.001; C=0.001; D=0.001	8.2 ± 0.4 8.6 (6.5–9.8)	8.7 ± 0.6 8.1 (6.1–10.6)	0.617

A = lean control (L-C) versus obese control (O-C), B = lean IGF1 (L-IGF1) versus obese IGF1 (O-IGF1), C = L-C versus O-IGF1, D = L-IGF1 versus O-C.

^aReported as mean ± S.E.M. and median (first quartile to third quartile). ^bCows used to collect embryos for gene expression analysis. ^cCows used to collect embryos for immunostaining.

This hypothesis on embryo quality needs to be substantiated with embryo transfer studies.

An important feature of this superovulated bovine model is that the detrimental microenvironment created by the intraovarian IGF1 injection in lean cows and the lack of effects in obese cows resemble to a certain extent the situation observed in PCOS, where IGF1 bioavailability is increased in normal-weight PCOS women, but reduced in obese PCOS patients (Silfen *et al.* 2003, Premoli *et al.* 2005, Pasquali & Gambineri 2008). In a natural high IGF1 microenvironment such as PCOS, oocytes and preimplantation embryos will be exposed continuously to high concentrations of IGF1, possibly exacerbating the effects observed in the present study. Research in mice has shown that *in vitro* exposure of preimplantation embryos to high IGF1 concentrations results in increased apoptosis and resorption rates after transfer to recipients (Chi *et al.* 2000, Pinto *et al.* 2002, Eng *et al.* 2007). Increases in the number of apoptotic cells and aberrant cell allocation have been observed in *in vitro*-produced bovine blastocysts exposed to high IGF1 concentrations from the zygote stage onwards (Velazquez *et al.* 2011). Results from the present *in vivo* model and the available *in vitro* information strongly indicate that high IGF1 concentrations are detrimental to the establishment of pregnancy. This information is not only relevant for PCOS women, but also for individuals using GH (Sonksen 2001), IGF1 (Guha *et al.* 2009), or bovine colostrum (Shing *et al.* 2009) to improve physical performance.

In conclusion, oocyte developmental competence was reduced by a relatively short exposure to supraphysiological concentrations of IGF1 in lean cows to the levels observed in obese cows. Superovulated obese cows seem to be refractory to IGF1 at the ovarian level. The adverse consequences on oocyte competence in lean cows and the absence of effects in obese cows after IGF1 intraovarian administration resemble to a certain extent the situation observed in PCOS patients, where IGF1 bioavailability is increased in normal-weight

PCOS women, but reduced in obese PCOS patients. This supports our proposal of using the superovulated female bovine as an alternative model for research on reproductive disorders in humans associated with abnormal IGF1 concentrations (Velazquez *et al.* 2009). Overall, the present data suggest that conditions or therapies inducing excessive IGF1 signaling should be avoided during the periconceptual period in order to reduce the risk of an early pregnancy loss in monovulatory species such as cattle and humans.

Materials and Methods

Animals

The study was carried out in the experimental herds of the Institute of Farm Animal Genetics (FLI), Mariensee, Germany. A total of 33 lactating Holstein Friesian and 44 non-lactating Deutsche Schwarzbunte (DSB) cows were used as embryo donors. Lactating cows were fed a mixed ration and additional concentrate based on milk yield. All lactating cows (lean cows) were in mid-lactation and had a BCS (five-point scale of Edmonson *et al.* (1989) 1 = emaciated and 5 = grossly overfat) of 2.5–3.5. This is the recommended BCS range for this lactation period to ensure that animals are not in a negative energy balance (Chagas *et al.* 2007). Deutsche Schwarzbunte non-lactating cows (obese cows) were fed for maintenance requirements and had not been lactating for at least 6 months. These animals had a BCS ≥ 4 (Fig. 5). Our experimental herd consists of Holstein cows that have in their genetic origin the Deutsche Schwarzbunte breed and in fact are crosses to various extents (50–100%) with DSB. Our long-term records indicate that both types of cows (Holstein and DSB) do not show differences regarding embryo production when subjected to superovulation. The mean age of all cows was 4 years. All cows were kept in tie-stalls with water *ad libitum*. Animal experiments were performed according to the German law for the protection of animals. The study comprised of two trials, in the first trial (February–November 2008) animals (lactating = 19 and non-lactating = 10) were superovulated in a cross-over design and in the second trial (June–December 2009) a different set of animals (lactating = 14

Figure 5 (A) Example of lactating lean and non-lactating obese cows used in this study. (B and C) Diagram showing animal allocation in both trials and experimental procedures performed in each cow (see text for details).

and non-lactating=34) were superovulated either as controls or IGF1-treated cows (Fig. 5). In the second trial, in the lactating lean category, seven cows were used in each group (i.e. control or IGF1), whereas in the non-lactating obese category 16 and 18 cows were used in the control and the IGF1-treated group respectively.

In vivo production of embryos

All animals were cycling regularly prior to use in the experiments. Transvaginal ultrasound-guided ovum pick-up and intraovarian injections were performed as previously described (Oropeza *et al.* 2004). For each cow, the experimental period started with aspiration of all visible antral follicles ≥ 5 mm in both ovaries at unknown stages of the estrous cycle (day 0) (Baracaldo *et al.* 2000). Two days after follicle ablation, a single intraovarian injection of $1 \mu\text{g}$ recombinant human IGF1 (291-G1; R&D Systems, Wiesbaden-Nordenstadt, Germany) diluted in 0.5 ml PBS (A0964; AppliChem, Darmstadt, Germany) supplemented with 0.1% BSA (A9647, Sigma-Aldrich) was administered in the stroma of each ovary. Control animals received only 0.5 ml PBS/BSA without IGF1. Any visible follicle with more than 4 mm in size on day 2 was aspirated immediately prior to intraovarian injection to ensure a homogenous population of follicles with 3–4 mm in diameter. Immediately after intraovarian treatment, cows entered a superovulatory regime with 500 μg porcine FSH combined with 100 μg porcine LH (Stimufol; Ulg, FMV PhR, University of Liege, Sart-Tilman, Belgium) in a total volume of 10 ml administered i.m. in 8 decreasing doses over 4 days. A prostaglandin $F_{2\alpha}$ analog (Estrumate; Intervet, Unterschleißheim, Germany) was applied on days 4 (pm) and 5 (am) followed by artificial insemination (AI) on days 6 (pm) and 7 (am–pm). All cows were inseminated three times with the same bull of proven fertility for AI. Eight days after first insemination (day 14 of the experimental period) embryos were recovered by non-surgical uterine flushing with 500 ml PBS supplemented with 1% (v/v) newborn calf serum (B15-001; PAA Laboratories GmbH, Pasching, Austria; Fig. 5). Prior to embryo recovery, the ovarian response (i.e. number of corpora lutea) was analyzed by transrectal ultrasonography (Bungartz & Niemann 1994), followed by collection of non-diluted ULF samples, as recently described (Velazquez *et al.* 2010). Blood samples were collected immediately after embryo collection from the coccygeal vein into tubes containing EDTA (Greiner

Bio-one, Frickenhausen, Germany). Blood samples were centrifuged (2000 g for 20 min) followed by plasma collection and storage at -25°C . Embryos were graded according to the IETS guidelines (Stringfellow & Seidel 1998) and processed within 30 min after collection. To discriminate between oocytes and zygotes, presumptive oocytes were subjected to differential cell staining (Velazquez *et al.* 2011). Animals in the cross-over design (first trial) were submitted again to follicular ablation and superovulation after at least one normal estrous cycle. Blastocysts of quality 1 (Stringfellow & Seidel 1998) collected from the first trial were used for gene expression analysis, whereas those collected in the second trial were used for immunostaining. All embryos were processed while maintaining the identity of the donor.

mRNA isolation, RT and quantitative real-time PCR

Blastocysts used for gene expression analysis were washed three times in 0.1% (w/v) polyvinyl alcohol (PVA)/PBS solution and placed individually into 0.6 ml siliconized Eppendorf tubes with $\sim 4 \mu\text{l}$ PVA/PBS and stored at -80°C until mRNA extraction. Poly (A) $^{+}$ RNA was isolated from single blastocysts and reverse transcribed into first-strand cDNA in a 0.2 ml reaction tube containing 20 μl reaction mixture as previously described (Niemann *et al.* 2010, Velazquez *et al.* 2011). Tubes with the reaction mixture containing sterile water instead of mRNA preparation were used as controls for contamination. Tubes with the reaction mixture and 2 μl (1 pg) rabbit globin mRNA and 9 μl sterile water were prepared to produce pool of cDNA globin for quantification of globin expression used for data normalization (see below). To perform real-time PCR, wells from 96-well optical reaction plates were loaded with 20 μl PCR mixture containing 10 μl power SYBR green PCR master mix (4367659; Applied Biosystems, Darmstadt, Germany), 0.8 μl (5 μM) each of the forward and reverse primers of the respective genes of interest (Table 4), 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; Velazquez *et al.* 2011). 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

Table 4 Primers used for real-time PCR.

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

(1:5) of cDNA from 60 blastocysts and pooled globin respectively (Niemann *et al.* 2010, Velazquez *et al.* 2011). 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 standard during RNA extraction). To normalize data to embryo cell number, the relative abundance of each transcript in individual embryos was divided by the mean total cell number (Table 2) and multiplied by 100 (Block *et al.* 2008). Individual blastocysts were analyzed and each analysis was repeated 7–18 times.

Immunofluorescence

Immunostaining of TP53 and IGF1R was carried out with a validated protocol for bovine embryos (Velazquez *et al.* 2011). Briefly, zona-intact blastocysts were fixed (4% paraformaldehyde) and permeabilized (0.5% Triton X-100) followed by blocking with 10% newborn calf serum (B15-001, PAA Laboratories GmbH) for 1 h at room temperature. Blocked embryos were incubated overnight at 4 °C with the primary antibody. The TP53 and IGF1R antibodies (Rabbit polyclonal, 9282; Cell Signalling Technology, Inc., Frankfurt, Germany) were diluted 1:100 and 1:50 respectively (Rabbit polyclonal, PAI-14212; Dianova, Hamburg, Germany). Embryos were then incubated with the Alexa Fluor 488-conjugated secondary antibody (1:100; Invitrogen, Ltd) for 2 h in the dark at room temperature. Embryos from different cows were incubated in drops of equal volume obtained from the same antibody preparation. Embryos were counterstained with propidium iodide (PI, 50 µl/ml, P4170; Sigma–Aldrich, Steinheim, Germany) and mounted individually on glass microscope slides with vectashield antifade mounting medium (H-1000; Vector Laboratories, Burlingame, CA, USA). *In vitro*-produced embryos (Velazquez *et al.* 2011) not incubated with the primary antibody served as negative controls. Embryos were analyzed by confocal laser-scanning microscopy (CLSM) with a Zeiss LSM 510 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) using a Plan-Apochromat 20×/0.75 objective. Alexa Fluor 488 and PI were detected with Argon (excitation wavelength at 488 nm) and Helium (excitation wavelength at 543 nm) lasers respectively. Optical sections of

1.98 µm thickness were made at 4 µm intervals through the whole embryo and analyzed with the LSM Image Browser software (Carl Zeiss MicroImaging GmbH). The total cell number was recorded. All embryos were scanned under the same confocal settings. 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. Fluorescence intensity is reported as signal strength as previously described (Velazquez *et al.* 2011).

Analysis of IGF1 in plasma and ULF

ULF samples were homogenized in 200 µl PBS including a protease inhibitor cocktail (complete mini, Roche Diagnostics) with ceramic beads using a MagnaLizer (Roche) and directly placed on ice. For IGF1 extraction, 200 µl HCl/ethanol (12.5 ml 2 M HCl/87.5 ml absolute ethanol) was added to 20 µl plasma or uterine fluid (20 µl from the 200 µl homogenization buffer solution plus the unknown amount of uterine fluid from the uterus) and placed on a shaker for 30 min at room temperature. TRIS buffer (100 µl; 5.5 g in 50 ml sterile water) were added and vortexed vigorously. The extract was centrifuged at 10 000 *g* for 15 min at 4 °C and frozen at –20 °C overnight. The next day, 200 µl were taken from the supernatant and dried for 1 h in a SpeedVac concentrator to remove the ethanol. The lyophilizate was then diluted with 200 µl sterile water, from which 20 µl were taken for analysis with ELISA. Samples were measured in duplicates from one extraction. Standards and samples were diluted in assay buffer containing 0.1% gelatin. Plates were coated with goat-anti-rabbit IgG (own production, Technical University of Munich) and blocked with 0.5% casein (Vector SP-5020). Rabbit polyclonal antibody (provided by Prof. Schams, Technical University of Munich) was added to the plate. Samples were incubated overnight at 6–8 °C. Thereafter, biotinylated IGF1 (Novozymes GroPep, Adelaide, Australia) was added and incubated for 6 h at 6–8 °C. Plates were then decanted, followed by addition of streptavidin–peroxidase conjugate solution (Sigma–Aldrich) in each well and incubation for 15 min at room temperature. After washing the plates four times, substrate solution (Sigma–Aldrich) was added. The reaction was stopped after 45 min using sulfuric acid (Sigma–Aldrich).

The absorption was photometrically determined at 450 nm. The lower detection limit was 200 pg/ml, and intra- and interassay coefficients of variation were <10%.

Analysis of leptin in plasma

The concentrations of leptin were measured by ELISA (Sauerwein *et al.* 2004) using ovine recombinant leptin (Gertler *et al.* 1998) as standard. The intra- and interassay coefficients of variations were 3.6 and 7.8% respectively. The minimal detection level of the assay was 0.3 ng/ml.

Statistical analysis

Data were analyzed with SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA). Parametric (*t*-test) and non-parametric (Mann–Whitney *U* test) tests were used as appropriate (i.e. depending on normality of data). Proportions were analyzed by χ^2 test. Associations between variables were tested by regression analysis. Differences in embryo yields were tested only in cows that responded to superovulation (i.e. two or more corpora lutea at the time of embryo recovery). Both mean $\pm$ S.E.M. and median are reported unless otherwise indicated. A value of $P \leq 0.05$ was considered to be statistically significant.

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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