Altered pregnancy outcomes in mice following treatment with the hyperglycaemia mimetic, glucosamine, during the periconception period

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Short running title: Periconceptional glucosamine and pregnancy outcome

Abstract

Exposure of cumulus-oocyte complexes to the hyperglycaemia mimetic, glucosamine during *in vitro* maturation impairs embryo development, potentially through up-regulation of the hexosamine biosynthesis pathway. This study examined the effects of *in vivo* periconception glucosamine exposure on reproductive outcomes in young healthy mice, and further assessed the effects in overweight, high fat-fed mice. Eight week old mice received daily glucosamine injections (20 or 400 mg/kg) for 3-6 days before and 1 day after mating (periconception). Outcomes were assessed at day 18 gestation. Glucosamine treatment reduced litter size, independent of dose. A high fat diet (21% fat) for 11 weeks prior to and during pregnancy reduced fetal size. No additional effects of periconception glucosamine (20 mg/kg) on pregnancy outcomes were observed in fat-fed mice. In mice fed control 6% fat diet glucosamine treatment at 16 weeks of age reduced fetal weight and increased congenital malformations. As differing effects of glucosamine were observed in 8-week and 16-week old control mice, maternal age effects were assessed. Periconception glucosamine at 8 weeks reduced litter size, while glucosamine treatment at 16 weeks reduced fetal size. Thus, *in vivo* periconception glucosamine exposure perturbs reproductive outcomes in mice, with the nature of the outcomes dependent upon maternal age.

Keywords: hexosamine biosynthesis pathway, hyperglycaemia, fetal development

Introduction

The link between hyperglycaemia and adverse reproductive outcomes has been extensively and well documented (Miller et al. 1981; Combs and Kitzmiller, 1991; Greene, 1999; McCance 2011; Simmons, 2011). Despite recent advances in therapeutic management there are still markedly greater rates of complications in pregnancies accompanied by hyperglycaemia, rather than normoglycaemia (Metzger *et al*. 2007; Kitzmiller *et al*. 2008; Shand *et al*. 2008; Ali and Dornhorst, 2011). The periconception period has recently emerged as a period of acute sensitivity to changes in the maternal metabolic environment. Negative effects of periconceptional maternal hyperglycaemia, diabetes and obesity on the developing oocyte and preimplantation embryo have been described, with consequences for pregnancy success, fetal development and postnatal outcomes (Jungheim and Moley 2008; Minge *et al*. 2008; Wyman *et al*. 2008; Jungheim 2010; Ramin *et al* 2010; Wang and Moley 2010; Cardozo *et al*. 2011; Fleming *et al*. 2012).

Glucosamine (GlcN), the simple amino sugar, has been documented to upregulate the hexosamine biosynthesis pathway, as it is converted to GlcN-6-phosphate, an intermediate of the pathway that bypasses the rate limiting glutamine-fructose-6-phosphate amidotransferase enzyme (GFAT) (Marshall *et al*. 1991; McClain and Crook, 1996). In somatic tissues, elevated flux through the hexosamine pathway has been associated with perturbed health states, including insulin resistance (Marshall *et al*. 1991; Patti *et al*. 1999; McClain 2002; Buse 2006; Teo *et al*. 2010). Previous studies have demonstrated that exposure of murine, bovine and porcine cumulus-oocyte complexes (COCs) to GlcN during *in vitro* maturation significantly reduces subsequent embryo developmental competence (Sutton-McDowall *et al*. 2006; Kimura *et al*. 2008; Schelbach *et al*. 2010). Similarly, GlcN addition during murine embryo culture impairs blastocyst development (Pantaleon *et al*. 2010), while addition of GlcN to bovine embryo culture, from the 8-cell stage, decreases the development rate of embryos and skews the sex ratio towards males (Kimura *et al*. 2008). During maturation of the COC, significant glucose flux through the hexosamine biosynthesis pathway supports expansion of the cumulus matrix (Sutton-McDowall *et al*. 2004), as UDP-N-acetyl glucosamine, the end product of the pathway, is the substrate for hyaluronic acid synthesis. However, UDP-acetyl glucosamine also serves as the substrate for *O*-linked glycosylation of serine and threonine residues in a range of proteins (Torres and Hart 1984). Hyperglycaemic disruption of cellular function is thought to be mediated through increased levels of *O*-linked glycosylation and altered function of key proteins as a result of upregulated

hexosamine biosynthesis pathway activity (Vosseller *et al*. 2002; Love and Hanover 2005; Butkinaree *et al*. 2010). Similarly, *in vitro* studies support a role for up regulation of the hexosamine biosynthesis pathway and perturbed *O*-linked glycosylation in mediating the negative effects of exposing COCs or early embryos to GlcN (McDowell *et al*. 2006; Pantaleon *et al*. 2010; Schelbach *et al*. 2010).

While the effects of *in vitro* exposure of developing oocytes and embryos to GlcN have been assessed, and studies have begun to investigate the mechanisms underlying these outcomes (McDowell *et al*. 2006; Pantaleon *et al*. 2010; Schelbach *et al*. 2010), the potential for *in vivo* effects of GlcN on oocyte and early embryonic development has been little considered. One small study of 54 women who used GlcN during pregnancy reported no adverse fetal effects (Sivojelezova *et al*. 2007). In mice, administration of GlcN at day 7.5 gestation has been shown to increase the incidence of neural tube defects (Horal *et al*. 2004), demonstrating that postimplantation exposure to GlcN can have negative effects in rodents. We therefore conducted studies to determine whether GlcN administration during the periconception period would affect reproductive outcomes in mice. Additional studies further assessed these effects in mice maintained on a high fat diet, to determine whether GlcN effects would be exacerbated in conditions of perturbed maternal metabolic state. These studies suggested the potential for the effects of GlcN on reproductive outcomes to be influenced by maternal age, therefore, the effects of periconception GlcN in mice of differing ages were also assessed.

Methods and Materials

Unless otherwise stated all chemicals were purchased from Sigma Chemical Co. (St Louis, USA).

Animals

Male and female C57Bl/6 mice were obtained from Laboratory Animal Services at The University of Adelaide. Mice were housed in the animal facilities at The Queen Elizabeth Hospital, Woodville, South Australia for Experiment 1 and at The University of Adelaide Medical School, Adelaide, South Australia, for Experiments 2 and 3. Mice were kept under 14:00-10:00 light-dark conditions and all females were first parity. Experiments were approved by the Animal Ethics Committees of Queen Elizabeth Hospital and The University of Adelaide.

***Experiment 1**: The effects of periconception GlcN treatment on pregnancy outcomes.*

GlcN treatment

Seven week-old naturally cycling female mice were weighed and divided into three, weight-matched groups. One week later, groups were randomly allocated to receive either 0, 20 or 400 mg/kg GlcN per day (for an association, 20 mg/kg equates to a daily dose of approximately 1500 mg in adult humans). Eight replicate experiments were performed with 3 mice per treatment group (total of 24 per treatment). Mice were weighed, and injected intraperitoneally with 5 μl per gram body weight of Dulbecco's PBS (0 mg/kg GlcN), or a solution of 4 mg/ml GlcN in PBS (20 mg/kg GlcN) or a solution of 80 mg/ml GlcN in PBS (400 mg/kg GlcN). Mice were injected for 3 consecutive days. All injections throughout the study were administered at 24 hour intervals. On the third day, each female was housed overnight with a male, and mating success was determined via the detection of a vaginal sperm plug the next morning. All mice were again treated with glucosamine or PBS on the fourth day. For successfully mated females this was the final injection. Females that did not mate were re-introduced to males and daily injections repeated until mating was achieved (followed by a final injection on the day following successful mating), or until mating had been attempted for a maximum of 4 nights, with those that did not mate excluded from the study. Therefore, females received between 4-7 glucosamine or PBS injections. Males were randomly allocated to females from different treatment groups throughout all replicates.

Pregnancy outcomes

On day 18 of pregnancy, mice were killed by cervical dislocation and post-mortem examinations were performed. The number of pregnant mice was recorded. Total fetuses and fetal resorptions were counted in each uterine horn. Litter size was defined as the total number of viable fetuses. Each fetus and its corresponding placenta were isolated, weighed, briefly examined for gross morphological appearance and classified as either normal or abnormal. Fetal crown-rump length was recorded.

***Experiment 2**: Periconception GlcN treatment in mice maintained on different fat content diets – effects on pregnancy outcomes.*

Dietary manipulation

Five-week-old female C57Bl/6 mice were weighed and randomly allocated into two groups, which were maintained on either a low fat (LF) ((w/w) 6% fat (SF04–057)) or high fat, high cholesterol diet (HF) ((w/w) 21% fat, 0.15% cholesterol (SF00–219)) diet

(Specialty Feeds, Glen Forrest, Australia) (Table 1). Twelve mice were allocated per treatment group and eight replicate experiments were performed (total of 96 mice per dietary group). Mouse weights were recorded weekly.

Plasma insulin and glucose tolerance

Blood samples were collected following 7, 10 and 11 weeks of dietary manipulation. Mice were fasted overnight and 400 – 600 µl blood was collected from anaesthetised mice via an orbital bleed. Blood was centrifuged for 10 min at 4^{o}C, and plasma was collected and stored at -20˚C. Plasma insulin levels were measured in duplicate using a Rat Insulin RIA Kit (LINCO Research, Inc., St. Charles, MO, USA), with a sensitivity of 0.1 ng/ml and an intra-assay coefficient of 1.4 – 4.6 %. Samples were collected from six mice per dietary group from two replicates, giving a total of 12 mice per dietary group.

Following blood collection at 10 weeks of dietary intervention, mice were allowed to recover from the anaesthesia for one hour. An intraperitoneal glucose tolerance test was then performed. Mice were injected intraperitoneally with 1 g D-glucose/kg bodyweight. Blood samples were collected by nicking of the tail vein, and blood glucose was measured using an Accu-Check® Advantage Glucometer at 0, 15, 30, 60, 90 and 120 minutes post-glucose injection. Glucose tolerance was assessed by calculating area under the glucose curve (Le Floch *et al*. 1990).

GlcN treatment

Following 11 weeks of dietary manipulation, mice were weighed and randomly assigned to receive either 0 or 20 mg/kg GlcN. This resulted in four treatment groups 1) LF – GlcN, 2) LF + GlcN, 3) HF – GlcN and 4) HF + GlcN with 48 mice per group. The average weight of all mice that had been on the low fat diet was used to determine the volume of a 4 mg/kg GlcN solution to correspond to a 20 mg/kg dose (i.e. 5 µl per kg bodyweight). This standardized volume was administered to all GlcN-treated mice in the LF and HF dietary groups, to avoid significant variation in total dosage due to different body weights. An equivalent volume of PBS was administered to control animals in LF and HF groups. Mice were injected with GlcN as described in Experiment 1, with the exception that females were placed with males for a maximum of 2 nights. Hence, mice in Experiment 2 were treated for 4-5 days with GlcN. Mice were maintained on their assigned LF or HF diets throughout pregnancy.

To determine whether GlcN treatment influenced glucose tolerance, mice that did not mate after two attempts were fasted overnight on the day of the final (fifth) GlcN injection.

Fasting blood samples were collected and glucose tolerance tests were performed as described above.

Pregnancy outcomes

Fetal and placental outcomes were assessed according to the methods described for Experiment 1. In addition, placental length and width were recorded to determine placental volume. Ovaries were also removed and corpora lutea (CL) numbers were counted as an indication of ovulation rate.

Experiment 3*: Periconception GlcN treatment of 8 and 16 week old mice – effects on pregnancy outcomes.*

GlcN treatment

Based on an observation of different effects of GlcN treatment in mice fed the control mouse chow diet in Experiments 1 and 2, the effects of maternal age at GlcN administration on reproductive outcomes were assessed. Five week old (young) and 13 week old (adult) mice were weighed weekly for 3 weeks. At 8 and 16 weeks of age, each age group was divided into weight-matched subgroups to receive either 0 or 20 mg/kg GlcN. This created 4 groups (8 wks - GlcN, 8 wks + GlcN, 16 wks - GlcN, and 16 wks + GlcN). Three mice were allocated to each treatment group, and eight replicates were performed (total of 24 mice per treatment group).

For each replicate, average weights for the 8 week old and 16 week old mice were used to calculate the volume of 4 mg/kg GlcN solution to correspond to a 20 mg/kg dose for each age group (as per Experiments 1 and 2). An equivalent volume of PBS was administered to control animals (0 mg/kg GlcN) in each of the age groups. Mice received injections of PBS or GlcN for a minimum of 4 and a maximum of 7 days as described in Experiment 1. Pregnancy outcomes were assessed at day 18 gestation as described for Experiment 2.

Statistics

Statistical analysis was performed with SAS (Statistical Analysis Software) version 9.2. Chi–squared analyses were performed for parameters relating to mating (n, %), pregnancy (n, %), viable day 18 fetuses (%), resorptions from implantations (%), fetuses with abnormalities (n, %) and pregnancies with abnormal fetuses (n, %). Comparison of the mean weekly weight change in mothers across all replicates for both experimental groups was performed with a Repeated Measures ANOVA and Bonferroni corrected

pairwise comparison. Parameters relating to fetal and placental size, litter size, implantation number and resorption number were assessed with a One-way ANOVA in Experiment 1 and a Two-way ANOVA in Experiments 2 and 3 using a mixed model analysis. In Experiments 2 and 3, CL number was also analysed via Two-way ANOVA. In addition, serum insulin levels and area under the curve was assessed by Two-way ANOVA. Bonferroni post hoc tests were performed for all ANOVAs. All mixed model analyses were performed both with and without the inclusion of co-variates, including days of GlcN exposure, maternal weight at pregnancy, litter size, implantation number and resorption number. Any effects of covariates on outcomes are reported and unless otherwise stated, reported statistics are unadjusted for covariates.

Results

Experiment 1*: The effects of periconception GlcN on pregnancy outcomes.*

Pregnancy rates

GlcN treatment did not affect the number or proportion of mice that mated, as indicated by the presence of a sperm plug. Similarly, there were no differences between treatment groups in the number of mice that were pregnant on day 18, or the proportion of mice that were pregnant relative to those that successfully mated (Table 2).

Litter size

Regardless of dose administered, GlcN treatment reduced the number of implantations and litter size (number of viable fetuses) at day 18 gestation ($P < 0.05$). The number of resorptions detected at day 18 pregnancy was not altered by GlcN treatment, but when expressed as a percentage of implantations, resorption rate was increased by GlcN treatment, regardless of dose (Table 2).

Fetal outcomes

Periconception GlcN treatment did not alter fetal weight or length of the viable fetuses at day 18 of pregnancy (Table 2) but tended to increase placental weight ($P = 0.08$). Birth defects, as determined by gross morphological appearance on day 18 gestation were not associated with any treatment. A single fetus presented with one very small eye in the control group, and another fetus had an abdominal hernia in the GlcN 20 mg/kg group.

Experiment 2*: Periconception GlcN treatment in mice maintained on different fat content diets.*

Effects of dietary manipulation on bodyweight and glucose tolerance prior to GlcN administration

Female mice maintained on the HF diet were heavier than their LF counterparts from5 weeks of dietary manipulation (LF diet: 21.1 ± 0.2 g (n=96); HF diet: 23.6 ± 0.3 g (n=96)) ($P < 0.05$). The weight difference increased, such that mice fed the HF diet were 22% heavier than the LF fed group by the time of mating at 11 weeks of dietary exposure (LF diet: 24.8 ± 0.3 g (n=96); HF diet: 30.5 ± 0.4 g (n=96)) ($P < 0.05$)).

After ten weeks of high fat feeding, there was no significant difference in glucose tolerance, assessed as glucose area under the curve, for mice on the LF and HF diets (LF

diet: 2402 ± 154 mM/ 120 min (N=12); HF diet: 2663 ± 124 mM/ 120 min (N=12)). There were also no differences detected in plasma insulin levels for mice maintained on the two diets at either 7 weeks (LF: 0.26 ± 0.06 ng/ml (n=10), HF: 0.23 ± 0.04 ng/ml (n=7)), 9 weeks (LF: 0.22 ± 0.04 ng/ml (n=9), HF: 0.24 ± 0.04 ng/ml (n=9)) or 10 weeks (LF: 0.28 ± 0.09 ng/ml (n=8), HF: 0.30 ± 0.08 ng/ml (n=9)) of dietary exposure. Insulin levels were measured in 12 mice from each dietary group at each time point, and were below the levels of detection for the assay (0.1 ng/ml) in 2-5 mice per group.

Glucose tolerance following GlcN administration

Glucose area under the curve in a glucose tolerance test, in non-pregnant mice, was not altered by 5 days GlcN treatment and did not differ between dietary groups (mM/ 120 min) (LF – GlcN: 2893 ± 95 (n = 10), LF + GlcN: 2807 ± 128 (n = 12), HF – GlcN: 3005 ± 112 (n = 12), HF + GlcN: 3080 ± 128 (n = 12)).

Pregnancy rates

Mice in this experiment were placed with males for a maximum of 2 nights, resulting in lower proportions mating when compared to Experiments 1 or 3. GlcN treatment did not affect the number or proportion of mice that mated. However, more LF mice that were treated with GlcN became pregnant than those maintained on a LF diet and not given GlcN ($P < 0.05$) (Table 3). In addition, less GlcN treated HF fed mice became pregnant after mating, when compared to GlcN treated LF fed mice, or when compared to HF fed mice that were not treated with GlcN ($P < 0.05$) (Table 3).

Litter size

Implantation number, litter size and resorption rates at day 18 of pregnancy were not affected by GlcN treatment or high fat feeding (Table 3). Ovulation rate, as indicated by the number of corpora lutea present at day 18 gestation, was also not affected by any treatment (Table 3).

Fetal outcomes

Fetal weight at day 18 of pregnancy was reduced by maternal high fat feeding ($P < 0.05$, Table 4). Periconceptional exposure to GlcN also reduced fetal weight ($P < 0.05$, Table 4). Although there was no significant interaction between the two factors, fetal weight was reduced by GlcN only in LF-fed mice. Exposure to a HF maternal diet also reduced fetal length ($P < 0.05$, Table 4), while periconception GlcN exposure did not affect fetal length (Table 4). Placental weight did not differ between treatment groups (Table 4). Placental

volume tended to be reduced in mothers fed a HF diet (P = 0.08), but there were no differences in placental volume between GlcN treatment groups (Table 4).

An increased number (P < 0.001) and proportion (P < 0.01) of fetuses presented with congenital abnormalities in the LF + GlcN group, when compared to all other groups (Table 4). Similarly, the number (P < 0.01) and proportion (P < 0.001) of pregnancies containing a fetus with abnormalities was higher for the LF + GlcN group relative to all others (Table 4). The proportion of mothers that carried birth-defected fetuses was also increased by exposure to a HF diet, irrespective of GlcN treatment, when compared to the LF – GlcN group (Table 4). The majority of abnormalities were eye defects (LF+GlcN: n=14/17 fetuses; HF-GlcN: n=3/4 fetuses; HF+GlcN: n=2/3 fetuses), characterised by missing one or both eyes or having underdeveloped eyes. Identification of defects was performed by gross examination only, no further histopathological analysis of the ophthalmic defects was performed. An abdominal omphalocele was present in 3 fetuses from the LF + GlcN group, and 1 fetus from each of the HF - GlcN and HF + GlcN groups.

Experiment 3*: Periconception GlcN treatment of 8 and 16 week old mice.*

Because different effects of GlcN treatment were observed in mice fed the control mouse chow diet between Experiments 1 and 2, we decided to investigate the effect of maternal age at periconceptual GlcN administration (8 wk vs. 16 wk) on reproductive outcomes, as this was the only major difference between the experimental groups. Older mice were heavier than younger mice for the 3 weeks they were housed prior to GlcN treatment (bodyweight at mating at 8 wk: 22.1 ± 0.2 g (n=24); bodyweight at mating at 16 wk: 25.0 ± 0.4 g (n=24) (P < 0.05)).

Pregnancy rates

GlcN treatment and maternal age did not affect the number or proportion of mice that mated or became pregnant (Table 5). Significantly more mice mated over the first 2 days compared to the third and fourth days (data not shown, P < 0.0001).

Litter size

An interaction effect was detected between GlcN and maternal age for the number of implantations and litter size (P < 0.01 for both), where GlcN administration in 8 wk old mice reduced implantations and litter size, but not in 16 wk old mice (GlcN x age interaction, P < 0.01 for both) (Table 5). Implantation number tended to be reduced by GlcN in 16 wk old mice, when compared to 16 wk mice that did not receive GlcN, but this

was not significant. Older mice that were not treated with GlcN had higher implantation rates and litter sizes than young mice ($P < 0.01$) (Table 5).

Overall, the total number of implantations ($P < 0.0001$) and viable fetuses ($P < 0.0001$) was lowest in the 8 wk + GlcN group. No differences in the number of resorptions were seen between the four groups (Table 5), however, a greater proportion of implantations resorbed ($P < 0.05$) in 8 wk old GlcN treated mice (Table 5). Mean ovulation rate, as determined by CL number, tended to be decreased in young mice that received 20 mg/kg GlcN, although this was not significant ($P = 0.076$) (Table 5).

Fetal outcomes

Maternal age did not independently influence fetal weight whereas GlcN was found to have a significant effect on fetal weight ($P < 0.05$), and there was a significant interaction between GlcN and maternal age ($P < 0.01$). Fetal weight was reduced by periconception GlcN treatment in 16 wk old mice ($P < 0.05$) (Table 6), while the same treatment did not affect fetal weight in 8 wk old mice.

GlcN reduced fetal length in pups derived from 16 wk old GlcN treated mice ($P < 0.05$) (Table 6). For fetal length, a significant interaction was observed between GlcN and maternal age ($P < 0.04$), that was lost when maternal weight was included in the analysis as a co-variate, suggesting that maternal weight may be a contributing component to age-associated changes in fetal size.

Placental weight was increased in 8 wk old mice treated with GlcN, when compared to all other groups ($P < 0.05$, Table 6). There was no effect of maternal age on placental weight. No differences were detected in placental volumes between the 4 groups (Table 6).

A significantly higher number of abnormal fetuses were present in litters of 16 wk old GlcN treated mice, compared to all other groups ($P < 0.05$, Table 6). A higher number, and proportion, of GlcN treated older mice had pregnancies characterized by at least one abnormally developing fetus, when compared to the remaining groups ($P < 0.05$, Table 6).

Birth defects were again characterised by eye defects (8 wk – GlcN, n=2 fetuses; 16 wk – GlcN, n=3 fetuses; 16 wk + GlcN, n=7 fetuses). One fetus in the 16 wk + GlcN group presented with an omphalocele.

Discussion

Previous studies have demonstrated that *in vitro* exposure of the developing oocyte or preimplantation embryo to glucosamine has detrimental consequences for subsequent embryo development (Sutton-McDowall *et al*. 2006; Kimura *et al*. 2008; Pantaleon *et al*. 2010; Schelbach *et al*. 2010). Furthermore, subcutaneous administration of 40 mg (~1600-2000 mg/kg) glucosamine to pregnant mice on day 7.5 days of gestation is associated with an increased incidence of neural tube defects (Horal *et al*. 2004). The current study has extended these observations by demonstrating adverse effects of *in vivo* glucosamine administration during the periconception period on subsequent murine embryonic and fetal development. These effects were manifested as reduced implantation rates, retarded fetal development and an increased incidence of congenital malformations, with the occurrence of these perturbations being influenced by accompanying maternal conditions.

Maternal age, in particular, was shown to influence the effects of periconceptional glucosamine on reproductive outcomes. Glucosamine treatment reduced litter size in young, 8-week old mice. The decrease in litter size was mediated in part by a reduction in mean implantation rate as well as an increase in the proportion of implantations that resorbed. In contrast, in older, adult mice (16 weeks of age), periconception glucosamine did not alter litter size, but reduced fetal weight and increased the incidence of congenital abnormalities. Furthermore, periconceptional glucosamine increased placental weight in 8-week, but not 16-week old, mice.

Mechanisms involved in the generation of divergent reproductive outcomes as a result of exposure to periconception glucosamine in different aged mice are unclear and require further investigation. In younger mice, implantation rate was decreased. Reduced litter size and the observed increase in placental weight may have contributed to maintenance of fetal size in glucosamine treated young mice. While implantation rates were not altered in older mice, reduced fetal weight and an increased incidence of birth defects does suggest that oocyte or embryo quality has been affected by glucosamine exposure at both ages, but with differing consequences for subsequent development. The periconception glucosamine treatment protocol used in the current study may potentially affect the preovulatory, ovulated or fertilizing oocyte, the early embryo or the uterine environment and receptivity. No significant difference in ovarian corpora lutea numbers suggests that the reduction in litter size in young mice is not due to fewer oocytes being ovulated. Body weight is higher in adult, compared to young mice, but body composition was not assessed in the current study. Whether changes in adiposity or metabolic indices could

have influenced the metabolism or actions of glucosamine at different ages is unclear. Body weight differences would result in a minor difference in total glucosamine dose administered on a per kg body weight basis, however, adverse effects were observed in the young mice, that would have received the lower total dose. Age related changes in activity of the hexosamine signalling pathway have been suggested; with one study reporting an increase in hexosamine biosynthesis pathway activity in aged rats (Einstein *et al*. 2008), while others report reductions in skeletal muscle activity of the GFAT enzyme and reduced levels of *O*-linked N-acetyl glucosamine (*O*-GlcNac) and *O*-GlcNAc transferase (OGT) in heart in mice and rats, across a similar age span to that assessed in the current study (Buse *et al*. 1997; Fulop *et al*. 2007). Previous *in vitro* studies have supported a role for glucosamine induced increases in flux through the hexosamine biosynthesis pathway, and an associated increase in *O*-linked glycosylation in the cumulus-oocyte-complex or early embryo, in mediating the adverse effects of glucosamine (Pantaleon *et al*. 2010; Schelbach *et al*. 2010). Further studies are required to assess the specific mechanisms through which short-term, periconceptional glucosamine administration is affecting oocyte and embryo developmental competence *in vivo*, including assessment of the hexosamine biosynthesis pathway and *O*-linked glycosylation, and to more fully characterise the interaction with maternal age.

The metabolic perturbations accompanying diabetes and obesity are associated with upregulation of the hexosamine biosynthesis pathway (Robinson *et al*. 1995; Buse *et al*. 1997; Considine *et al*. 2000; Veerababu *et al*. 2000, Kaneto *et al*. 2001; McClain 2002) and both conditions are associated with an increased incidence of adverse pregnancy outcomes, including miscarriage, congenital abnormalities and altered fetal growth (Miller *et al.* 1981; Combs and Kitzmiller 1991; Greene 1999; Stothard *et al.* 2009; Simmons, 2011). Studies have demonstrated negative effects of exposing the developing oocyte or embryo to maternal hyperglycaemia *in vivo* or *in vitro* (Diamond *et al*. 1989; Moley *et al*. 1991; Wyman *et al*. 2008; Jungheim and Moley 2008; Wang *et al*. 2009; Ramin *et al*. 2010; Wang and Moley 2010) and improved glycemic control during the pre- and early pregnancy period in women with diabetes has been associated with reduced incidence of miscarriage and risk for fetal abnormalities (Ray *et al*. 2001; Temple *et al*. 2006). Furthermore, embryos collected at the one-cell stage from diabetic mice, and transferred to normoglycemic recipients, have an increased incidence of retarded fetal growth and fetal abnormalities (Wyman *et al*. 2008), providing clear evidence that exposure to perturbed maternal glucose metabolism during the periconceptional period can affect subsequent fetal development.

In the current study, we therefore further assessed the effects of periconception glucosamine in overweight mice that had been maintained on a high fat diet, to assess glucosamine effects under perturbed metabolic conditions which may be associated with upregulation of the hexosamine pathway. Increased fasting serum insulin, glucose and free fatty acids, and increased adipose tissue weight, have previously been reported following 16 weeks exposure to the same high fat diet used in the present study (Minge *et al*. 2008). However, high fat fed mice in the current study did not demonstrate differences in glucose tolerance or fasting insulin, following 11 weeks dietary intervention, suggesting that longer dietary exposure may have been required to induce detectable impairments in insulin and glucose metabolism. Specific assessment of insulin sensitivity may have also been required to more fully characterise the effects. Nevertheless, fat fed mice were overweight suggesting that some degree of metabolic perturbation did occur and the observed effects of the high fat diet on fetal size support this. Litter size was not altered in high fat fed mice, however, in agreement with previous studies, feeding a high fat diet before and throughout pregnancy reduced fetal weight and length (Jungheim *et al*. 2010), irrespective of glucosamine treatment. These findings extend a previous study reporting adverse effects of this diet on blastocyst development (Minge *et al*. 2008). Periconception glucosamine treatment reduced fetal weight in 16 week old mice fed control chow; however, no additive effects of glucosamine treatment and high fat feeding on reproductive outcomes were observed. Other studies have reported that glucosamine administration, or upregulation of the hexosamine biosynthesis pathway through overexpression of the GFAT enzyme, does not potentiate high fat feeding induced insulin resistance in rats or mice (Barrientos *et al*. 2010; Cooksey and McClain 2010), suggesting a potential for maximal upregulation of the pathway. The lack of additive effects may also implicate a role for the hexosamine biosynthesis pathway in mediating the negative effects of periconception high fat diets on oocyte and embryo competence, however, specific analyses of hexosamine biosynthesis pathway activity and its downstream effectors in reproductive tissues from obese mice are required to assess this further.

Animal studies have demonstrated reductions in insulin sensitivity following intravenous administration of glucosamine (Rossetti *et al*. 1995; Virkamaki *et al*. 1997; Patti *et al*. 1999; Stampinato *et al*. 2003). Despite some studies suggesting similar effects in humans, systematic reviews of clinical studies have identified limited evidence for an effect of oral glucosamine on insulin sensitivity or glucose tolerance in human subjects, but acknowledge the need for further study in subjects with existing risk of impaired glucose metabolism (Anderson *et al*. 2005; Dostrovosky *et al*. 2011; Simon *et al*. 2011). The current study did not detect any differences in glucose tolerance in non-pregnant

mice following 5 days of intraperitoneal glucosamine administration, but insulin sensitivity was not assessed. Nevertheless, despite the lack of measurable effects of acute glucosamine administration on glucose or insulin levels, effects of glucosamine on reproductive outcomes were observed, suggesting that the effects of short-term glucosamine have occurred independent of measurable metabolic perturbations.

In mice, *in vivo* uptake of radiolabelled glucosamine has been detected in follicular fluid, and cells of the ovarian follicle, suggesting that glucosamine is not metabolised exclusively externally to the reproductive system, and indicating the potential for tissue specific effects, distinct from peripheral metabolic effects (Fowler and Guttridge 1987; Fowler 1988; Fowler and Barrett 1989; Horal *et al*. 2004). Bioavailability of GlcN is dependent upon its route of administration (Aghazadeh-Habashi *et al*. 2002). Intraperitoneally injected GlcN has been shown to have complete bioavailability, resembling intravenous administration (Aghazadeh-Habashi *et al*. 2002), while bioavailability of orally administered glucosamine is low (Setnikar *et al*. 1993; Adebowale *et al*. 2002; Aghazadeh-Habashi *et al*. 2002; Persiani *et al*. 2005), Intraperitoneal injection of glucosamine in the current study allowed administration of a consistent, bioavailable glucosamine dose. However, while the dose of 20 mg/kg (0.5 mg in a 25 g mouse) used in this study resembles the commonly recommended oral dose of 1500 mg in an adult human, differences in the route of administration may result in a higher bioavailable dosage in this study. Nevertheless, in contrast to a previous study reporting adverse effects of postimplantation treatment with high doses of glucosamine (Horal *et al*. 2004), the current study has utilised treatment within a physiologically relevant range.

Periconceptional glucosamine treatment was associated with an increase in placental weight in young mice. The effects of maternal metabolic perturbation on placental development are complex and dependent on the timing and nature of the perturbation. For example, feeding mice a diet high in fat and sugar throughout pregnancy reduces fetal and placental weight (Vaughan *et al*. 2012), while no effect on placental weight, but an increase in fetal weight, has been reported in high fat fed mice (Jones *et al*. 2009). Transient hyperglycaemia in early-mid gestation in the rat (day 10) has been reported to increase placental weight (Ericsson *et al*. 2007). However, studies assessing the effects of periconceptional metabolic perturbations (eg. Wyman *et al*. 2008) have generally not reported on placental outcomes. Further assessment of placental structure and nutrient transport capacity would be required to determine whether increased placental weight in glucosamine treated young mice was associated with altered function. Similarly, fetal weight was reduced, while placental size was unaffected, in mice fed high fat diets

throughout pregnancy in the current study. Whether altered placental structure or function contributed to reduced fetal growth in fat-fed mice requires further analysis.

Abnormalities observed following exposure of oocytes and preimplantation embryos to hyperglycaemia included neural tube defects, limb deformities and growth retardation (Wyman *et al*. 2008). Similarly, common malformations associated with diabetic pregnancies are heart, neural tube and caudal defects (Salbaum and Kappen 2011). The defects observed in the current study did differ, with primarily ocular defects, and some incidences of fetal omphalocele. Preconception obesity has been associated with an increased risk of fetal omphalocele (Waller *et al*. 2007) and glucose transporter-1 deficient mice exhibit increased rates of microphthalmia, suggesting that metabolic perturbations could contribute to the abnormalities. The strain of mice (C57Bl/6) used in this study do have an increased susceptibility to ophthalmic defects (Sulik *et al*. 1981; Parnell *et al*. 2006). However, acute alcohol exposure in early gestation increases the incidence of ocular defects in offspring of these mice, suggesting that perturbations during embryonic development can increase the risk (Sulik *et al*. 1981; Parnell *et al*. 2006).

Advanced maternal age (Hansen 1986; Friede *et al*. 1988; Nybo Andersen *et al*. 2000; Miletic *et al*. 2002; Hsieh *et al*. 2010) and impaired maternal glucose metabolism (McCance 2011; Simmons 2011) are recognized as independent factors that perturb reproductive success rates. The increasing rate of pregnancy in older women (Martin *et al*. 2006, Chan *et al*. 2009) and the increasing incidence of metabolic disorders involving perturbed glucose metabolism (Dunstan *et al*. 2002; Colagiuri *et al*. 2005; Wang *et al*. 2011), suggests that the potential for interactive effects should be considered. Studies that have considered the effects of intercurrent illness on adverse perinatal outcomes in older women, to date, have suggested that the effects of older age are independent of any existing diabetes (Jacobsson *et al*. 2004; Delbaere *et al*. 2007). Nevertheless the observation that reproductive outcomes were perturbed in adult mice of an age well within the reproductively fit range in combination with a metabolic perturbation that did not result in measurable differences in glucose metabolism, suggests that the potential for interaction should be considered.

Conclusion

Collectively these results demonstrate that *in vivo*, periconception glucosamine exposure in mice elicits detrimental effects upon fetal development that are dependent on maternal age. Adverse effects of increased maternal weight, induced by high fat feeding, on fetal development were also confirmed, but these effects were not exacerbated by glucosamine treatment. The specific effects at a cellular level that contribute to altered

reproductive outcomes associated with acute periconception glucosamine exposure require further study. Given that hyperglycemia elicits effects by stimulating divergent pathways, glucosamine may prove to be a useful tool to study the specific effects of this signalling pathway in glucose induced pathophysiologies and for further analysing associations between age and hyperglycemia. Previous studies have suggested dysregulation of the hexosamine biosynthesis pathway as a potential mechanism through which maternal hyperglycaemia elicits effects on oocyte and preimplantation embryo development (Schelbach *et al*. 2010; Pantaleon *et al*. 2010). The current study, suggests that further studies should assess the potential role of increased activity of the hexosamine biosynthesis pathway, and associated effects such as increased *O*-linked glycosylation, in contributing to the *in vivo* effects of periconceptional maternal hyperglycaemia.

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Table 1. Composition of control (low fat) and high fat diet

Ingredient	Control	High fat
	g/kg	
Casein	195	195
DL Methionine	3	3
Sucrose	341	341
Wheat Starch	306	154
Cellulose	50	50
Canola Oil	60	-
Clarified butter (Ghee)	-	210
Cholesterol	-	1.5
Calcium carbonate	17.1	17.1
Sodium chloride	2.6	2.6
Potassium citrate	2.5	2.6
Potassium dihydrogen phosphate	6.9	6.9
Potassium sulphate	1.6	1.6
AIN93G trace minerals	1.4	1.4
Choline chloride (65%)	2.5	2.5
SF00-219 vitamins	10	10
Etoxyquin (66%)	0.04	0.04
	Calculated values	
Protein (%)	19	19
Total Fat (%)	6	21
Fibre (%)	9.4	9.4
Digestible Energy (MJ/kg)	16.1	19.4
% Calculated energy from lipids	21	40

High fat diet (SF00-210), Control diet (SF04-057) (Specialty Feeds, Glen Forrest, Western Australia)

Table 2. Effect of periconception GlcN treatment (20 or 400 mg/kg) on reproductive outcomes at day 18 gestation

	0 mg/kg GlcN	20 mg/kg GlcN	400 mg/kg GlcN
Mated (n) (%)	22/24 (82%)	19/24 (70%)	18/24 (67%)
Pregnant (n) (%)	13/22 (59%)	14/19 (74%)	12/18 (67%)
Implantations (n)	7.2 ± 0.2^{a}	5.4 ± 0.3^{b}	4.2 ± 0.6^{b}
Litter size (n)	6.0 ± 0.5^{a}	3.1 ± 0.4^{b}	2.5 ± 0.7^{b}
Resorptions (n)	1.0 ± 0.3	1.1 ± 0.2	1.6 ± 0.3
Viable d18 fetuses from implantations (%)	85.4%	70.6%	62.9%
Resorptions from implantations (%)	$15.7\%^{a}$	$29.4\%^{b}$	$38.7\%^{b}$
Fetal weight (mg)	792 ± 22	741 ± 42	852 ± 46
Fetal length (mm)	18.7 ± 0.3	17.4 ± 0.6	19.9 ± 0.8
Placental weight (mg)	80.2 ± 1.1	87.8 ± 2.1	85.3 ± 2.9

Implantations represents the mean number of implantation sites (sum of viable and non-viable fetuses and resorption sites) detected per mouse at day 18 gestation. Litter size represents the number of viable fetuses at day 18 gestation. Fetal and placental data represent n=74 (0 mg/kg), n=38 (20 mg/kg) and n=36 (400 mg/kg). Data presented as mean ± sem. Values with different superscripts are significantly different, $P < 0.05$.

Table 3. Effect of high fat feeding and periconception GlcN treatment (20 mg/kg) on reproductive outcomes at day 18 gestation

	LF - GlcN	LF + GlcN	HF - GlcN	HF + GlcN
Ovulation rate	10.4 ± 0.6	9.3 ± 0.1	9.1 ± 0.2	11.2 ± 1.1
Mated (n) (%)	28/48 (58%)	28/48 (58%)	25/48 (52%)	26/48(54%)
Pregnant (n) (%)	12/28 (43%)^{a,c}	20/28 (71%)^{b}	15/25 (60%)^{a,b}	8/26 (31%)^{c}
Implantations (n)	8.8 ± 0.9	9.1 ± 0.6	7.6 ± 0.8	9.0 ± 0.5
Litter size (n)	7.0 ± 0.8	8.2 ± 0.6	7.1 ± 0.8	8.2 ± 0.6
Viable d18 fetuses from implantations (%)	86.1%	88.0%	88.9%	91.4%
Resorptions (n)	1.2 ± 0.3	1.1 ± 0.3	0.8 ± 0.3	0.8 ± 0.2
Resorptions from implantations (%)	13.0%	12.0%	11.1%	8.6%

Ovulation rate represent the number of corpora lutea present on the ovary at day 18 gestation. Litter size represents the number of viable fetuses at day 18 gestation. Data presented as mean ± sem. LF = mice maintained on a control diet, HF = mice maintained on a high fat diet. Values with different superscripts are significantly different, $P < 0.05$.

Table 4. Effect of high fat feeding and periconception GlcN treatment (20 mg/kg) on fetal and placental outcomes at day 18 gestation

	LF - GlcN	LF + GlcN	HF - GlcN	HF + GlcN
Fetal weight (mg)	904 ± 55^{a}	761 ± 14^{b}	658 ± 14^{c}	680 ± 16^{c}
Fetal length (mm)	18.0 ± 0.2^{a}	18.8 ± 0.2^{a}	17.9 ± 0.2^{b}	17.7 ± 0.2^{b}
Placental weight (mg)	74.7 ± 1.4	73.1 ± 1.0	72.5 ± 1.3	73.1 ± 1.6
Placental volume (mm^2)	123 ± 6	134 ± 6	118 ± 6	117 ± 6
Fetuses with abnormalities	0^{a}	17^{b}	4^{c}	3 ^{ac}
Proportion abnormal from total fetuses	0/99 (0%)^{a}	17/161 (11%)^{b}	4/88 (5%)^{a}	3/74 (4%)^{a}
Pregnancies with abnormal fetus	0^{a}	9^{b}	2^{a}	2^{a}
Proportion pregnancies with abnormal fetus	0/10 (0%)^{a}	9/20 (45%)^{b}	2/15 (13%)^{c}	2/8 (25%)^{c}

Data presented as mean ± sem. LF = mice maintained on a control diet, HF = mice maintained on a high fat diet. Data are from n=12 litters, 91 fetuses (LF - GlcN), n= 20 litters, 151 fetuses (LF + GlcN), n= 15 litters, 103 fetuses (HF - GlcN), n=8 litters, 74 fetuses (HF + GlcN). Values with different superscripts are significantly different, $P < 0.05$.

Table 5. Effect of maternal age at mating and periconception GlcN treatment (20 mg/kg) on reproductive outcomes at day 18 gestation

	8 wk - GlcN	8 wk + GlcN	16 wk - GlcN	16 wk + GlcN
Ovulation rate	11.4 ± 1.3	9.0 ± 2.6	12.2 ± 0.6	12.8 ± 0.7
Mated (n) (%)	20/24 (83%)	19/24 (79%)	21/24 (88%)	19/24 (79%)
Pregnant (n) (%)	11/20 (55%)	7/19 (37%)	9/21 (43%)	11/19 (58%)
Implantations (n)	8.0 ± 0.3[a]	6.1 ± 0.8[b]	10.1 ± 0.5[c]	8.5 ± 0.4[a c]
Litter size (n)	7.0 ± 0.3[a]	4.4 ± 0.6[b]	8.5 ± 0.5[c]	7.5 ± 0.6[a c]
Viable d18 fetuses from implantations (%)	86.5%	72.1%	84.6%	88.3%
Resorptions (n)	1.3 ± 0.2	1.7 ± 0.7	1.5 ± 0.3	1.0 ± 0.3
Resorptions from implantations (%)	13.5%[a]	27.9%[b]	15.4%[a]	11.7%[a]

Ovulation rate represent the number of corpora lutea present on the ovary at day 18 gestation. Litter size represents the number of viable fetuses at day 18 gestation. Data presented as mean ± sem. Mice were mated at 8 weeks (8 wk) or 16 weeks (16 wk) of age. Values with different superscripts are significantly different, P<0.05.

Table 6. Effect of maternal age at mating and periconception GlcN treatment (20 mg/kg) on reproductive outcomes at day 18 gestation

	8 wk - GlcN	8 wk + GlcN	16 wk - GlcN	16 wk + GlcN
Fetal weight (mg)	851 ± 23[a]	827 ± 34[a]	866 ± 35[a]	746 ± 20[b]
Fetal length (mm)	19.6 ± 0.2[a]	19.6 ± 0.6[a]	19.5 ± 0.4[a]	18.6 ± 0.2[b]
Placental weight (mg)	74.7 ± 1.6[a]	88.0 ± 2.5[b]	79.1 ± 2.1[a]	80.8 ± 1.6[a]
Placental volume (mm^2)	128 ± 0.5	138 ± 3	126 ± 4	141 ± 5
Fetuses with abnormalities	2[a]	0[a]	3[a]	8[b]
Proportion abnormal from total fetuses	2/77 (3%)	0/31 (0%)	3/77 (4%)	8/83 (10%)
Pregnancies with abnormal fetus	2[a]	0[a]	2[a]	5[b]
Proportion pregnancies with abnormal fetus	2/11 (18%)[a]	0/7 (0%)[b]	2/9 (22%)[a]	5/11 (45%)[c]

Data presented as mean ± sem. Mice were mated at 8 weeks (8 wk) or 16 weeks (16 wk) of age. Data are from n=11 litters, 77 fetuses (8 wk - GlcN), n= 7 litters, 31 fetuses (8 wk + GlcN), n= 9 litters, 77 fetuses (16 wk - GlcN), n= 11 litters, 83 fetuses (16 wk + GlcN). Values with different superscripts are significantly different, $P < 0.05$.