Hyperglycaemia potentiates the teratogenicity of retinoic acid in diabetic pregnancy in mice

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Abstract

Aims/hypothesis. We recently showed in mice that maternal diabetes increases embryonic susceptibility to caudal regression induced by vitamin A metabolite retinoic acid. Here we tested whether in the maternal diabetic milieu hyperglycaemia is the critical factor responsible for mediating this increased susceptibility.

Methods. Non-diabetic pregnant mice were made hyperglycaemic by subcutaneous injections of glucose at regular intervals. Conversely, diabetic pregnant mice were treated with phlorizin to induce renal glucosuria and thus reduce blood glucose concentrations. Pregnant mice were treated with retinoic acid and the extent of caudal regression in mouse embryos, measured in terms of the ratio of tail length to crown–rump length was assessed. Embryos were also examined for *Wnt-3a* expression and cell death.

Results. Embryos of mice treated with glucose had a greater extent of caudal regression induced by retinoic

acid than saline-treated controls, with enhanced down-regulation of *Wnt-3a* expression and exacerbated cell death specifically at the caudal end of the embryo. Embryos of diabetic mice treated with phlorizin had a similar extent of caudal regression to embryos of non-diabetic mice after treatment with retinoic acid.

Conclusions/interpretation. Hyperglycaemia increases embryonic susceptibility to caudal regression induced by retinoic acid, with the underlying cellular and molecular changes closely mimicking those that occur in maternal diabetes. Reduction of blood glucose concentrations in diabetic mice completely abolishes this increased susceptibility to retinoic acid. These results suggest that in maternal diabetes hyperglycaemia is the critical factor responsible for potentiating the teratogenic effect of retinoic acid. [Diabetologia (2004) 47:515–522]

Keywords Caudal regression · Retinoic acid · Hyperglycaemia · Maternal diabetes · *Wnt-3a* · Cell death · Mouse embryo

Infants of mothers with diabetes mellitus are more prone to congenital malformation than those of non-diabetic pregnancies [1, 2]. As most malformations in

infants of diabetic mothers are due to developmental errors at a very early stage of pregnancy [3], a better understanding of the risk factors that predispose these embryos to malformation could lead to better pre-conceptual care to prevent anomalies.

Although it is evident that diabetic embryopathy results from multifactorial interactions [4], few investigations of the association between diabetic embryopathy and other factors like food or drugs taken during pregnancy have been undertaken. Using the mouse as a model, we recently reported that embryos of diabetic mothers are more susceptible to the teratogenic effect of retinoic acid, the bioactive metabolite of vitamin A, in developing caudal regression [5]. The increased severity of caudal regression in embryos of diabetic

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Abbreviations: RAR, retinoic acid receptors · RBP, retinol binding protein · CRABP, cellular retinoic acid binding proteins

mice after retinoic acid treatment was associated with enhanced down-regulation of *Wnt-3a*, a gene that is indispensable for the development of the caudal embryonic region, and exacerbated cell death specifically in the tail bud. However, it is not clear which factor or factors in the maternal diabetic milieu are responsible for the teratogenic interaction with retinoic acid.

In humans, effective glycaemic control during pre-conception and early gestation has generally led to a lower frequency of malformations [6, 7, 8]. Moreover, increased glucose concentrations in vivo and in vitro culture in excess glucose have been shown to be teratogenic in animal studies [9, 10, 11]. The purpose of our study, therefore, was to specifically test whether hyperglycaemia is the critical factor that increases embryonic susceptibility to caudal regression induced by retinoic acid.

Materials and methods

Animals. The colony of ICR mice at The Chinese University of Hong Kong was established from breeding pairs originally obtained from Harlan UK (Bicester, Oxon, UK) in 1998. The mice were kept under a 12:12 h light-dark cycle in a humidity-controlled room and fed with irradiated LabDiet for rodents (PMI Nutrition, Richmond, Ind., USA) and ozone-sterilised tap water.

The principles of laboratory animal care set forth by the NIH were followed and approval of all animal experimentation procedures was obtained from the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Induction of hyperglycaemia. Timed matings were set up between 8- to 10-week-old non-diabetic female and non-diabetic male ICR mice. Starting from day 8 of gestation, female mice received subcutaneous injections of 1 ml of 25% D-glucose dissolved in PBS at 1.5-h intervals over a period of 16.5 h. Control female mice received equivalent injections of PBS. Glucose concentrations in blood from the tail vein were measured using the Glucometer Elite (Bayer, Newbury, UK) every 1.5 h. At the midpoint of glucose treatment, i.e. hour 8 on day 8 of gestation, pregnant mice received an intraperitoneal injection of 25 mg/kg body weight of all-*trans* retinoic acid (Sigma, St. Louis, Mo., USA) suspended in peanut oil, whereas control mice received an equivalent volume of suspension vehicle. The mice were killed at day 10.5 of gestation and embryos were removed from the uterus for analysis. To determine the extent of caudal regression, tail length (defined as the length of the caudal region from the posterior limit of the hindlimb bud to the end of the tail) and crown-rump length were measured using an eyepiece graticule on a stereomicroscope.

Phlorizin treatment. Diabetes was induced in 7- to 8-week-old female ICR mice by injecting streptozotocin to kill the insulin-producing pancreatic beta cells. This was done two weeks before mating as described previously [5]. Female mice were classified as diabetic if blood glucose concentrations were greater than 16.5 mmol/l. Untreated age-matched female ICR mice served as non-diabetic controls (blood glucose concentration: 5.0–7.7 mmol/l).

Diabetic or non-diabetic female mice were mated with non-diabetic male ICR mice. To induce renal glucosuria, starting from hour 12 on day 7 of gestation, pregnant mice received three intraperitoneal injections of 0.35 g/kg body weight of

phlorizin (Sigma), dissolved in 40% propylene glycol (Sigma), or an equivalent volume of suspension vehicle as control. Injections were given at 9-h intervals. At hour 8 on day 8 of gestation mice were treated with 25 mg/kg retinoic acid. The tail length and crown-rump length of embryos were recorded at day 10.5 of gestation as described above.

Detection and semi-quantitation of *Wnt-3a*. To find out whether hyperglycaemia increased embryos' susceptibility to caudal regression induced by retinoic acid via similar molecular and cellular changes as observed in the interaction of retinoic acid with maternal diabetes, embryos were removed from the uterus 16 h after retinoic acid treatment, in order to examine the expression of *Wnt-3a* in the tail bud. Whole embryos were subjected to in situ hybridisation using digoxigenin-labelled probes [12, 13], whereas the tail buds of embryos from the same litter were pooled together and subjected to semi-quantitative RT-PCR analysis. Polymerase chain reaction was carried out using forward (5'-GAA TCT CTA TGG GGA CTG CTA GG) and reverse (5'-GTA TTA AGT GTC AGA GCG GAT GC) primers to amplify an 82 bp sequence corresponding to nucleotide sequences 2107–2127 and 2188–2166 of mouse *Wnt-3a*. Forward (5'-GAG ATT ACT GCT CTG GCT CC) and reverse (5'-GGA CTC ATC GTA CTC CTG C) primers were used to amplify a 150 bp sequence corresponding to nucleotide sequences 868–887 and 1017–999 of mouse β -actin, which served as a positive control for mRNA preparation, and for semi-quantitative analysis of *Wnt-3a* mRNA expression. We did 30 PCR cycles, comprising: denaturation at 94°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 30 s. To ensure the cycle number was within the logarithmic phase, three tenfold serial diluted cDNA samples were used. The band volumes of *Wnt-3a* and β -actin were measured from digitised images using Quantity One quantitation software (Bio-Rad, Hercules, Calif., USA).

Whole-mount staining of dying cells. To compare the extent of cell death in the tail bud region of embryos under different treatments, the supravital dye Nile blue sulphate was used to stain dead cells in whole-mount preparations 24 h after treatment with retinoic acid or suspension vehicle, according to the protocol described previously [14].

Statistical analysis. Data on blood glucose concentrations were analysed by Student's *t* test. Data on tail length, the ratio of tail length to crown-rump length and relative intensity of *Wnt-3a* to β -actin expression were analysed by one-way ANOVA, followed by the Bonferroni test and the Contrast test, using SPSS software (SPSS, Chicago, Ill., USA), with statistical significance set at a *p* value of less than 0.05.

Results

Hyperglycaemia in early stages of organogenesis increases embryonic susceptibility to retinoic acid. Injections of glucose at regular intervals 8 h before and 8 h after retinoic acid injection increased the blood glucose concentration from 6.8 ± 0.2 mmol/l to a diabetic level of 17.9 ± 3.2 mmol/l at the time of retinoic acid injection ($p < 0.01$). Without retinoic acid, the embryos of mice treated with glucose showed no morphological differences from the PBS-treated controls (Fig. 1a). In these two groups of embryos the tail length (data not shown) and the ratio of tail length to crown-rump

Fig. 1a–d. Effect of hyperglycaemia on susceptibility to caudal regression induced by retinoic acid. Morphological appearance (a–c) of embryos of PBS-treated mice (PBS) and glucose-treated mice (GLU) when exposed to 25 mg/kg retinoic acid (RA) or suspension vehicle as control (CON). * marks the position of the hindlimb bud; the broken line marks the caudal limit of the tail. Note the striking caudal regression in embryos exposed to hyperglycaemia and RA (c). Scale bar: 1.5 mm and 0.5 mm in magnified caudal regions. Caudal regression reflected by reduction in the tail length to crown–rump length (TL/CRL) ratio (d) in embryos of different treatment groups. Values represent means $\pm$ SEM. Analysis was by one-way ANOVA followed by Bonferroni test. Number of embryos: PBS(CON), $n=110$; GLU(CON), $n=136$; PBS(RA), $n=127$; GLU(RA), $n=108$. $^{\#}p<0.05$ vs PBS(CON); $^{\dagger}p<0.001$ vs GLU(CON); $^{\ddagger}p<0.001$ vs PBS(RA)

length were very similar (Fig. 1d). Retinoic acid reduced tail length (data not shown) and the ratio of tail length to crown–rump length in both groups of embryos, but with a much greater reduction ($p<0.001$, analysed by one-way ANOVA followed by Contrast test) in embryos of mice treated with glucose

Fig. 2a–e. Effect of hyperglycaemia on expression level of *Wnt-3a*. In situ hybridisation (a–d) for *Wnt-3a* in embryos of PBS-treated mice (PBS) and glucose-treated mice (GLU) 16 h after treatment with 25 mg/kg retinoic acid (RA) or suspension vehicle as control (CON). The red arrowhead and yellow arrow mark the expression of *Wnt-3a* in the tail bud and neural tube respectively. Between 44 and 58 embryos were examined in each treatment group. The embryo of the PBS(RA) treatment group with the lowest observed expression level of tail bud *Wnt-3a* is shown in (c). *Wnt-3a* expression was extinguished in the tail bud of 20% of GLU(RA) embryos, as shown in a representative embryo (d). Scale bar: 0.5 mm. The graph (e) shows the relative intensity of *Wnt-3a*: β -actin PCR products. Values represent means $\pm$ SEM. Analysis was by one-way ANOVA followed by Bonferroni test. Number of litters: PBS(CON), $n=8$; GLU(CON), $n=3$; PBS(RA), $n=9$; GLU(RA), $n=6$. $^{\#}p<0.001$ vs GLU(CON); $^{\dagger}p<0.001$ vs PBS(RA)

(Fig. 1c,d) than in the PBS-treated controls (Fig. 1b,d). Hence, embryos developing under hyperglycaemic conditions have increased susceptibility to caudal regression induced by retinoic acid.

*Exposure to retinoic acid under hyperglycaemic conditions led to enhanced down-regulation of *Wnt-3a* and exacerbated cell death in the tail bud.* Our examination of expression of *Wnt-3a* and the extent of cell death in the embryos found that exposure to hyperglycaemic

Fig. 3a–d. Effect of hyperglycaemia on cell death. Nile blue sulphate staining of dead cells in the caudal region of embryos of PBS-treated mice (PBS) and glucose-treated mice (GLU) 24 h after treatment with 25 mg/kg retinoic acid (RA) or suspension vehicle as control (CON). Dead cells appear dark blue, the arrowhead marks the position of the tail bud. Cell death was moderately increased in the PBS(RA) tail bud (c), but markedly increased in the GLU(RA) tail bud (d). Representative embryos from the 12 to 15 examined in each treatment group are shown. Scale bar: 0.2 mm

conditions alone did not alter *Wnt-3a* expression in the tail bud of embryos in comparison with the PBS-treated controls (Fig. 2a,b,e). In contrast, after the injection of retinoic acid, *Wnt-3a* was down-regulated to a greater extent in the tail bud of embryos of mice treated with glucose (Fig. 2d,e) than in the tail bud of the PBS-treated controls (Fig. 2c,e; $p < 0.001$, analysed by one-way ANOVA followed by Contrast test). In 20% of the embryos of mice treated with glucose ($n = 58$), *Wnt-3a* expression was completely extinguished in the tail bud 16 h after retinoic acid treatment (Fig. 2d), while the remaining 80% of these embryos had a very low level of *Wnt-3a* mRNA transcripts. However, in the group treated with PBS and retinoic acid ($n = 44$), clearly detectable *Wnt-3a* mRNA transcripts occurred even in embryos with the lowest *Wnt-3a* expression level (Fig. 2c). The difference in *Wnt-3a* expression levels between the embryos of the PBS-treated and glucose-treated groups was restricted to the tail bud, with expression in the neural tube being unaffected by retinoic acid treatment (Fig. 2c,d), an observation that closely resembled the effect of maternal diabetes on *Wnt-3a* expression in embryos treated with retinoic acid.

Analysis of cell death by whole-mount supravital dye staining showed that cell death was not observed in the tail bud of embryos of mice treated with glucose or PBS alone (Fig. 3a,b). In contrast, 24 h after retinoic acid treatment, there were more dying cells in the tail bud of embryos in the group treated with glucose than in the PBS-treated controls (Fig. 3c,d). Maximum apoptosis was observed 24 h after retinoic acid treatment with lower levels before and after this time

Fig. 4a–d. Effect of reducing blood glucose on susceptibility to caudal regression. Morphological appearance (a–c) of embryos of retinoic acid-treated non-diabetic mice (ND) and retinoic acid-treated diabetic mice (SD) that had been exposed to phlorizin (PHZ) or suspension vehicle as control (CON). * marks the position of the hindlimb bud; the broken line marks the caudal limit of the tail. Phlorizin treatment completely rescued caudal regression in embryos exposed to retinoic acid within a diabetic milieu (c). Scale bar: 1.5 mm and 0.5 mm in magnified caudal regions. Caudal regression reflected by reduction in the tail length to crown–rump length (TL/CRL) ratio (d) in retinoic acid-treated embryos of different treatment groups. Analysis was by one-way ANOVA followed by Bonferroni test. Values represent means $\pm$ SEM. Number of embryos: ND(CON), $n = 98$; SD(CON), $n = 77$; ND(PHZ), $n = 118$; SD(PHZ), $n = 99$. # $p < 0.001$ vs ND(CON); † $p < 0.001$ vs SD(CON)

point. Taken together, these findings indicate that the increased extent of caudal regression in embryos treated with retinoic acid under conditions of hyperglycaemia is associated with the same molecular and cellular changes as in maternal diabetes.

Reduction of blood glucose concentrations completely abolishes the increased susceptibility to caudal regression induced by retinoic acid. After ascertaining that increased blood glucose interacts with retinoic acid in a similar way to maternal diabetes, we next tested whether the reduction of blood glucose concentrations could abolish the teratogenic interaction of maternal diabetes with retinoic acid. Administration of phlorizin to non-diabetic mice did not alter blood glucose concentrations or induce any morphological abnormalities (Fig. 4a), whereas our preliminary studies found that blood glucose concentrations of diabetic mice began to drop 12 h after phlorizin treatment. Hence, we started the phlorizin regime at hour 12 on day 7 of gestation, which led to a reduction ($p < 0.001$) in the blood glucose concentrations from 32.7 ± 0.3 mmol/l before phlorizin treatment to 11.4 ± 0.6 mmol/l at the time of retinoic acid injection (hour 8, day 8). With this reduction in blood glucose concentrations, the caudal region of embryos of retinoic acid-treated diabetic mice injected with phlorizin (Fig. 4c) became morphologically indistinguishable from embryos of retinoic acid-treated non-diabetic mice (Fig. 4a). This was in marked contrast to the embryos of retinoic acid-treated diabetic control mice injected with vehicle, which had severe caudal regression (Fig. 4b). The tail length (data not shown) and the ratio of tail length to crown-rump length (Fig. 4d) of embryos of retinoic acid-treated diabetic mice injected with phlorizin were so much increased that there was no difference between the diabetic and non-diabetic mice treated with retinoic acid (Fig. 4d). Taken together, these results indicate that the increased susceptibility to caudal regression induced by retinoic acid in diabetic pregnancy can be completely abolished by lowering blood glucose concentrations to near normal levels.

Discussion

In this study, we show that induction of hyperglycaemia during the early stages of organogenesis in non-diabetic mice can mimic maternal diabetes by increasing embryonic susceptibility to caudal regression induced by retinoic acid. Apart from increasing blood glucose concentrations, the repeated subcutaneous injection of D-glucose probably also affects other metabolic pathways and leads to an increase in insulin concentrations. Nevertheless, the increased susceptibility to retinoic acid in embryos of streptozotocin-induced insulin-deficient diabetic mice was completely abolished by selective reduction of blood glucose concentrations via specific inhibition of renal tubular reabsorption of glucose using phlorizin [15]. Thus, irrespective of possible changes in other metabolic pathways, our findings strongly suggest that hyperglycaemia in the maternal diabetic milieu is the critical factor that interacts with retinoic acid.

Several studies have reported multiple abnormalities in embryos developing under conditions of hyperglycaemia induced either by in vitro culture in serum supplemented with glucose, or by in vivo injection of glucose [9, 10, 11]. In contrast, we found that embryos of mice treated with glucose closely resembled embryos of PBS-treated control mice. The lack of apparent morphological abnormalities in embryos treated with glucose suggests that in the present study hyperglycaemia in itself was probably not teratogenic. In fact, although hyperglycaemia down-regulates developmentally important genes and induces cell death in tissues such as the neural tube [10], we found no change in the expression level of *Wnt-3a*, nor any increase in cell death in the tail bud under hyperglycaemic conditions alone. Yet, when exposed to retinoic acid, embryos developing under conditions of maternal hyperglycaemia had enhanced down-regulation of *Wnt-3a* and exacerbated cell death in the tail bud, compared with embryos developing under normoglycaemic conditions. This suggests that the interaction between hyperglycaemia and retinoic acid is not additive, but rather hyperglycaemia potentiates the teratogenicity of retinoic acid.

What are the mechanisms by which the teratogenicity of retinoic acid is increased in mice under hyperglycaemic conditions? The extent of caudal regression induced by retinoic acid, in non-diabetic mice, is dependent on developmental stage [14]. Some studies have shown that streptozotocin-induced diabetic rat embryos are retarded in growth and development [16]. Others, however, report that mouse embryos of streptozotocin-induced diabetic mothers or embryos cultured in serum supplemented with high glucose are not developmentally delayed or growth-retarded [5, 11, 17]. We examined embryos 24 h after the beginning of glucose injections and found no differences in morphology or somite number compared with PBS-treated control embryos, regardless of whether or not the mice had received retinoic acid (glucose-treated controls: 16.9 ± 0.3 somites; PBS-treated controls: 17.1 ± 0.6 ; glucose and retinoic acid-treated: 17.3 ± 0.3 ; PBS and retinoic acid-treated: 17.1 ± 0.5 ; unpublished data by one of the authors). Hence, developmental delay is unlikely to account for the increased susceptibility of embryos from hyperglycaemic pregnancy to caudal regression induced by retinoic acid.

The teratogenic effect of retinoic acid is highly dose-dependent [14, 18], raising the possibility that the amount of retinoic acid delivered to the embryo or taken up by the cell could be enhanced under maternal hyperglycaemia, thereby increasing the incidence of malformations induced by retinoic acid. Blood flow to the uterine and decidual tissues is increased during the early stages of pregnancy in diabetic rats [19], suggesting that retinoic acid could be delivered to developing embryos at an increased concentration under conditions of hyperglycaemia. Retinoic acid in the

embryo is synthesised from vitamin A (retinol) obtained from the maternal circulation. Hence, a further level of regulation of retinoic acid bioavailability can be provided by the retinol binding protein (RBP), the cellular retinol binding proteins I and II, and the cellular retinoic acid binding proteins (CRABP) I and II, which are expressed in the yolk sac, placental tissues and also in highly regulated patterns in the embryo [20, 21, 22, 23]. In fact, levels of RBP are reduced in the plasma, liver and kidney of streptozotocin-induced diabetic rats, diabetic Biobreeding (BB) rats and Type 1 diabetic patients [24, 25, 26, 27]. It is not known whether levels of the various retinoid binding proteins are altered in placental and embryonic tissues under hyperglycaemic conditions, which could affect the availability of retinoic acid to specific embryonic tissues, thereby enhancing the teratogenic effect of retinoic acid. For instance, CRABP sequesters free retinoic acid, thus preventing it from binding to the retinoic acid receptors (RAR) in regions of the embryo, such as the tail bud, where normal development requires low retinoic acid concentrations [22, 23, 28]. Hence, if CRABP levels are reduced in the embryo under conditions of hyperglycaemia, this could enhance the teratogenic effect of retinoic acid on the tail bud.

Alteration of the catabolism of retinoic acid provides a further level of regulation of retinoic acid teratogenicity. Retinoic acid plays an essential role in many developmental processes [29]. Both an excess and a deficiency of retinoic acid result in an overlapping array of malformations that affect the same target organ systems in the offspring [30, 31, 32], showing that the concentration of retinoic acid needs to be tightly regulated for normal development. The major retinoic acid catabolising enzyme is *CYP26*, which is expressed specifically in the caudal embryonic region, from the tail bud to the presomitic region [33], and catabolises retinoic acid that is synthesised and diffuses from the anterior somitic tissue [34]. Targeted disruption of *CYP26* in mice leads to caudal regression syndrome [28], with the extent of axial truncation closely resembling that induced by exogenous retinoic acid [14]. In mice and rats, *CYP26* mRNA expression is positively regulated by vitamin A [35]. Moreover, vitamin A levels are lowered in humans with Type 1 diabetes [27, 36, 37, 38] and in rodents with streptozotocin-induced diabetes [39]. It is possible, therefore, that a reduction of vitamin A in the plasma of diabetic mothers leads to reduced *CYP26* expression in the embryo, diminishing the embryo's ability to catabolise exogenous retinoic acid, and thereby enhancing retinoic acid teratogenicity.

Apart from affecting uptake, availability or catabolism of retinoic acid, maternal hyperglycaemia could also intervene in retinoic acid signalling by altering the efficacy of signal transduction. The action of retinoic acid is mediated via the formation of complexes

with nuclear RAR, which then bind to *cis*-acting retinoic acid response elements of target genes to activate, or sometimes inhibit their transcription. The several known RAR isoforms are expressed in spatially and temporally restricted embryonic domains [22, 23]. For instance, *RAR-γ* is expressed in the tail bud, but not in the neural tube [23]. Embryos homozygous for a loss-of-function mutation in *RAR-γ* are resistant to caudal regression induced by retinoic acid, showing that *RAR-γ* is involved in mediating the effect of retinoic acid in the tail bud [40]. Hence, it is possible that retinoic acid-induced down-regulation of *Wnt-3a* is mediated via *RAR-γ*, leading to specific reduction of *Wnt-3a* in the tail bud, but not in the neural tube. It has been shown that the expression levels of different RAR isoforms are reduced to various extents in embryonic tissues of animals deficient in vitamin A [41]. In adult tissues, interestingly, RAR isoforms whose expression is reduced to the greatest extent under conditions of vitamin A deficiency are induced several times more plentifully by exogenous retinoic acid than RAR isoforms that are not affected by vitamin A deficiency [42]. As reduced vitamin A availability is associated with diabetes [27, 36, 37, 38, 39], it is possible that the expression of RAR isoforms regulated by retinoic acid, e.g. *RAR-γ*, is reduced in embryos under maternal hyperglycaemia. If exogenous retinoic acid up-regulates embryonic *RAR-γ* to a greater extent in hyperglycaemic than in normoglycaemic conditions, this could result in enhanced down-regulation of *Wnt-3a*, specifically in the tail bud, as observed in this study.

In conclusion, our study shows that hyperglycaemia increases the susceptibility of mouse embryos to the teratogen, retinoic acid. This underlines the importance of tight glycaemic control during early diabetic pregnancy when the risk of congenital malformation is highest. It is known that vitamin A supplementation increases levels of retinoic acid in human plasma [43] and animal studies further show that vitamin A teratogenesis results from increased embryonic concentrations of retinoic acid [44]. Together with growing evidence that vitamin A levels are altered in diabetic patients [27, 36, 37, 38], which could thus affect the availability, catabolism or signal transduction of retinoic acid in the embryo as discussed earlier, our findings of an interaction between hyperglycaemia and retinoic acid suggest that, in addition to tight glycaemic control, close monitoring of maternal plasma vitamin A levels during preconception and early gestation may reduce the risk of congenital malformation in diabetic pregnancy.

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