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# Teratogenic effect of diabetic serum is prevented by supplementation of superoxide dismutase and N-acetylcysteine in rat embryo culture

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**Summary** Congenital malformations are more common in offspring of diabetic mothers than offspring of non-diabetic mothers. The precise cell biological mechanism leading to the increased incidence of congenital malformations in diabetic pregnancy is not known. In previous studies increased glucose and β-hydroxybutyrate concentrations were found to cause embryonic dysmorphogenesis. We have previously shown that rat embryos, cultured in serum from insulin-treated diabetic rats, develop malformations, despite normalisation of glucose and β-hydroxybutyrate concentration, thereby suggesting a multifactorial teratological nature of the diabetic environment. In the present study, therefore, we aimed to characterise the teratogenic activity of various components of diabetic serum and in addition to study the possible anti-teratogenic effects of supplementation of superoxide dismutase and N-acetylcysteine in rat embryo culture. We found that diabetic serum has a teratogenic effect on embryo development, a capacity residing in the alteration of several serum components in addition to glucose. Improving the embryonic capability to scavenge oxygen radicals, either by increasing superoxide dismutase activity or by supplying a rate-limiting precursor (N-acetylcysteine) for the enhanced synthesis of reduced glutathione, blocks the embryonic dysmorphogenesis. [Diabetologia (1997) 40: 7–14]

**Keywords** Diabetic pregnancy, embryo development, congenital malformation, glucose, β-hydroxybutyrate, branched chain amino acids, embryo culture, superoxide dismutase, N-acetylcysteine.

Diabetic embryopathy and growth retardation are approximately 2–3 times more common in infants of diabetic women than in offspring of non-diabetic pregnancy [1]. The mechanisms causing these developmental disturbances are unknown [2, 3]. Experimental studies in vivo and in vitro have suggested that glucose and β-hydroxybutyrate cause growth retardation and malformations in rodent embryos [4–6]. In an earlier study we showed that serum from insulin-treated diabetic rats is teratogenic despite normalisation of glucose and β-hydroxybutyrate concentrations [7], in line with similar results by Buchanan et al. [8]. These studies support the notion of a multifactorial aetiology of embryonic dysmorphogenesis in diabetic pregnancy [9, 10].

In the present work we have extended these studies by assessing the components in excess of high glucose and high ketone bodies that may exert significant teratogenic activity in vitro. This was done by culturing day 9 rat embryos for 48 h in serum from normal rats and rats made diabetic either with 50 mg/kg streptozotocin (STZ) or with 75 mg/kg STZ (denoted 50 MD and 75 MD). The glucose concentration of the culture media was set at 30 mmol/l,

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*Abbreviations*: GOT, Glutamic oxalacetic transaminase, EC 2.6.1.1; GPT, glutamic pyruvic transaminase, EC 2.6.1.2; GSH, reduced glutathione; N, normal, non-diabetic rat; NAC, N-acetylcysteine; + N, addition of NAC to culture medium; SOD, Superoxide dismutase; + S, addition of SOD to culture medium; STZ, streptozotocin; 50 MD or 75 MD, manifest diabetes in rat, induced by 50 mg/kg or 75 mg/kg STZ.

regardless of type of serum, and was kept at 10 mmol/l for one experimental group cultured in normal medium as a reference.

Based on earlier results [10–12] we also aimed to investigate a possible involvement of an excess of free oxygen radicals in the embryonic dysmorphogenesis elicited by a diabetic environment in vitro. This was done by supplementing the culture medium with either superoxide dismutase (SOD) or N-acetylcysteine (NAC). After the culture period, the effects on embryonic development were evaluated by morphologic examination of the embryos, as well as by measurements of embryonic protein and DNA content. In addition, a number of metabolites in the different sera were determined, in order to estimate possible relationships between disturbed metabolism of these compounds and altered embryonic development.

## Materials and methods

*Animals.* Rats from a local Sprague-Dawley-derived substrain (U rats) were used. The offspring of diabetic U rats exhibit an increased incidence of congenital malformations [13]. All rats were fed a commercial pelleted diet (AB Analysen, Lidköping, Sweden) and had free access to food and tap water. Female and male rats were caged overnight and a positive vaginal smear the following morning designated gestational day 0. The pregnant rats were killed on gestational day 9.

*Whole embryo culture.* The pregnant rats were killed by cervical dislocation on gestational day 9 between 12 h and 14 h and the embryos were prepared for embryo culture using the method of New [14]. The whole uterus was dissected out and transferred to a petri dish filled with 154 mmol/l NaCl. Each embryo with intact surrounding membranes was carefully dissected free of uterine and decidual tissue with watchmakers' forceps under a stereo microscope. The embryos were subsequently transferred to a 50-ml culture tube (Falcon 2070, Becton Dickinson, Lincoln Park, NJ, USA) with 4 ml rat serum and 1 ml saline with appropriate additions of glucose and antioxidants. Care was taken to mix embryos from different rat mothers in each culture tube.

The embryos, within their intact yolk sacs, were maintained in a roller incubator at 38 °C and 60 rev/min. At the start of culture, the tubes were gassed with 5 % $O_2$, 5 % $CO_2$ and 90 % $N_2$ (v/v/v) and capped tightly. After 24 h culture the heartbeat and general development of the conceptuses were inspected in a stereo microscope. The embryos were subsequently transferred to new culture tubes with fresh medium gassed with 20 % $O_2$, 5 % $CO_2$ and 75 % $N_2$ (v/v/v). The following morning (i. e. after a further 20 h of culture) the tubes were gassed with 40 % $O_2$, 5 % $CO_2$ and 55 % $N_2$ (v/v/v) for 10 min to obtain an appropriate oxygen tension for this stage of culture. After a further 6–8 h of culture, the embryos were dissected out of their yolk sacs and examined under a stereo microscope. The crown rump length, somite number and malformation score (assigning 0, 1, 5, or 10 points to no malformation, minor malformation, less severe, or severe malformation) of the embryos were determined. A malformation score of 0 indicated a completely normal embryo, fully rotated with a closed neural tube (Fig. 1A). Embryos given a score of 1 showed one minor, and only one, deviation from this pattern, mainly an open posterior end of the neural tube. A score of 5 signified one major, and only one, malformation, mainly an open neural tube in the rhombencephalon area or a slight tail twist, whereas a score of 10 indicated an embryo with multiple major malformations such as open neural tube, rotational defects, and/or heart enlargement (Fig. 1B and 2).

Each embryo was homogenized by ultrasound disruption (20 kHz, 60 W for 5 s) in 1 ml 0.5 M NaOH, and kept at + 4 °C until measurements of protein and DNA were performed. The protein content of the homogenates was determined by the method of Lowry et al. [15] using bovine serum albumin as standard, and DNA was measured as described by Kissane and Robins [16] and Hinegardner [17].

*Preparation of serum for embryo culture.* Retired male breeders (BK Universal, Sollentuna, Sweden), weighing 400–450 g, were used for serum preparation. The rats were divided in three groups, in two groups the rats were given a single i. v. injection of STZ, 50 or 75 mg/kg body weight (50 MD and 75 MD, respectively), whereas the rats of the third group did not receive any injection (denoted N). Three days later all rats with serum glucose concentration above 20 mmol/l (glucose analyser; Beckman Instruments, Fullerton, Calif., USA) were identified as manifestly diabetic (MD), whereas STZ-injected rats with lower serum glucose concentration were not used as serum donors. Before the STZ injection, the serum donors did not differ in body weight. At the time of serum harvest 4–5 days after the STZ injection, the 50 MD animals had largely maintained their body weight, whereas the 75 MD donors had lost 5–10 %.

Four or five days after the STZ administration, the MD rats were anaesthetised with ether, blood was collected from the abdominal aorta, and centrifuged immediately. The serum fraction was collected, supplemented with sodium benzylpenicillinate and streptomycin to give a final concentration of 60 and 100 mg/l, respectively, and stored at –20 °C until used. Blood from N rats was treated in the same way. On the day of culture serum was thawed, heat inactivated at 56 °C for 1 h, diluted with 20 % NaCl, and gassed with 5 % $O_2$, 5 % $CO_2$, and 90 % $N_2$ for 30 minutes immediately before use.

Measurements of metabolite and vitamin concentrations, as well as enzyme activity were performed on pooled batches of serum from 10–20 rats (N, 50 MD, or 75 MD), or pooled medium from 2–5 culture tubes of different culture conditions. Serum and culture media were characterized with respect to concentration of D-glucose [18], fructosamine [19], protein [20], cholesterol [21], triglycerides [22], β-hydroxybutyrate [23], creatinine [24], glutamic oxalacetic transaminase (GOT [ = AST], EC 2.6.1.1), glutamic pyruvic transaminase (GPT [ = ALT], EC 2.6.1.2) [25, 26] with the aid of a Cobas MIRA multichannel analyzer (Hoffman-La Roche, Basle, Switzerland). Most metabolites were measured using standard reagent kits from Hoffman-La Roche (kits nos. 07–3672–4 [glucose], 07–3669–4 [fructosamine], 07–3678–3 [protein], 07–3679–1 [cholesterol], 07–3663–5 [triglycerides], and 07–3667–8 [creatinine]). β-hydroxybutyrate was measured using a kit no. 310-A from Sigma (Sigma Diagnostics, St. Louis, Mo., USA), and the enzymes were measured using standard reagent kits from Hoffman-La Roche (kits nos. 07–3641–4 [GOT], and 07–3638–4 [GPT]).

For the determination of free amino acids, 500 μl aliquots of the serum samples were deproteinized by the addition of 150 μl aliquots of buffered sulphosalicylic acid [27] containing norleucine as internal standard. Prior to analysis the tubes were centrifuged at 10 000 × *g* for 5 min and loaded into the sample chain of a Biotronik LC-5001 amino acid analyzer

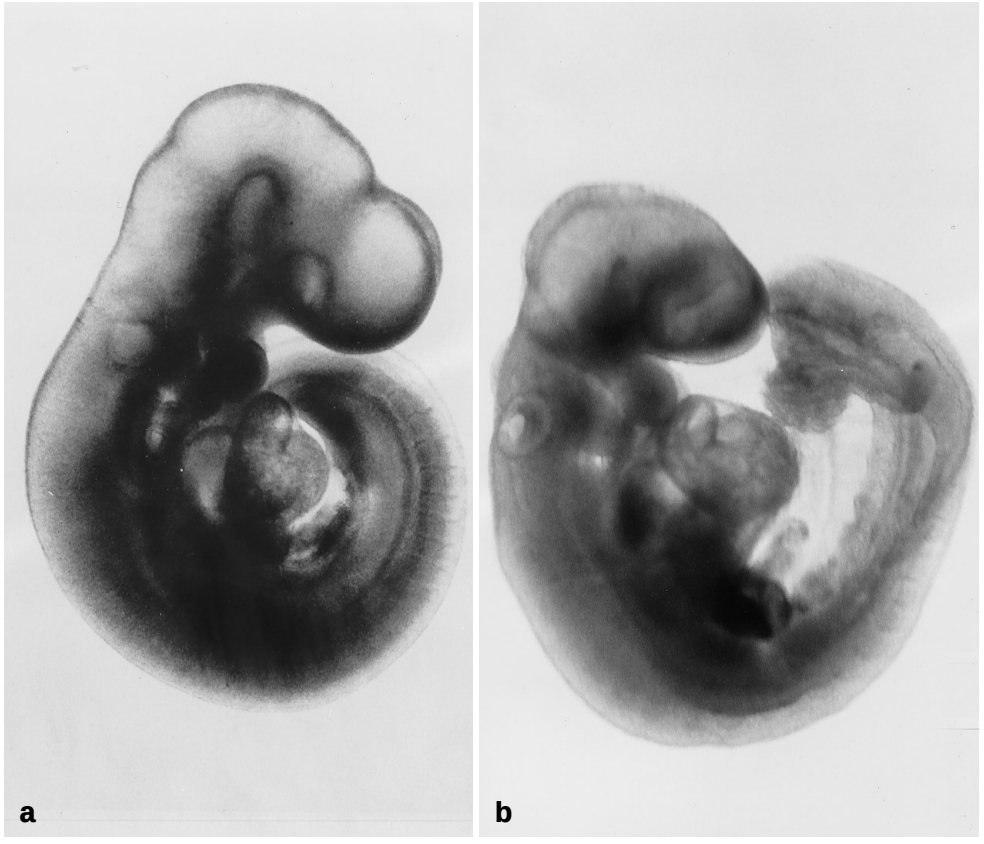


**Fig. 1 a, b.** Rat embryos after 48 h culture in vitro, exposed to 10 mmol/l glucose (**a**), or 30 mmol/l glucose (**b**). The low-glucose cultured embryo shows no malformations, closed neural tube, normal body curvature, a crown-rump length of 4 mm, and 30 somites (malformation score: 0). In contrast, the high-glucose cultured embryo shows multiple abnormalities, including open neural tube in several regions, slightly malrotated tail, enlarged heart, a crown-rump length of less than 3 mm and only 15 somites (malformation score: 10)

(Frankfurt-am-Main, Germany). The chromatograms were integrated using a two-channel Shimadzu CR2-AX integrator (Shimadzu Co., Kyoto, Japan).

Measurement of folate and vitamin $B_{12}$ was performed with the dual $B_{12}$/Folate RIA Kit CT 301 (Johnson & Johnson Clinical Diagnostics, Amersham, Buckinghamshire, UK). The serum samples were denatured together with potassium cyanide to release the vitamins from their binding proteins. The liberated vitamins competed subsequently with labelled vitamins for binding to hog intrinsic factor and folate binding protein, and the free fraction of the ligands was absorbed onto coated charcoal. The bound fraction was measured in a gamma counter and compared to a standard curve.

### *Statistical analysis*

Differences between means were evaluated by one-way analysis of variance (ANOVA) and the applied test was Fisher PLSD [28] at the 95 % significance level or chi square statistics (with Yates' correction), whichever was applicable [29].

## Results

The serum from the 50 MD and 75 MD rats differed from the N serum in several ways (Table 1). Between N and 50 MD we found that folate decreased, and that a number of amino acids were decreased (threonine, serine, glutamic acid, glutamine, proline, glycine, alanine, citrulline, methionine, tyrosine, phenylalanine, ornithine, lysine, arginine), whereas the branched chain amino acids were clearly increased (valine, isoleucine, leucine). The 75 MD serum differed from the N serum by a slightly increased vitamin $B_{12}$ concentration. The amino acid concentrations were more variable in the 75 MD serum compared to N serum, where a large number were decreased (taurine, aspartic acid, proline, citrulline), and in addition to the branched chain amino acids, also several amino acids were increased compared with N serum (threonine, asparagine, glutamic acid, glutamine, glycine, phenylalanine, ornithine, histidine, arginine). In particular, only arginine and the branched chain amino acids showed a clearcut dose-response effect with successively higher concentration with increased STZ dose (Table 1). The majority of the other amino acids showed a decrease in the 50 MD serum, and a return to, or an over-shoot of, the N value in the 75 MD serum.

The metabolite concentrations after the first and second 24 h culture are displayed in Table 2. The glucose concentrations remained close to the initial level

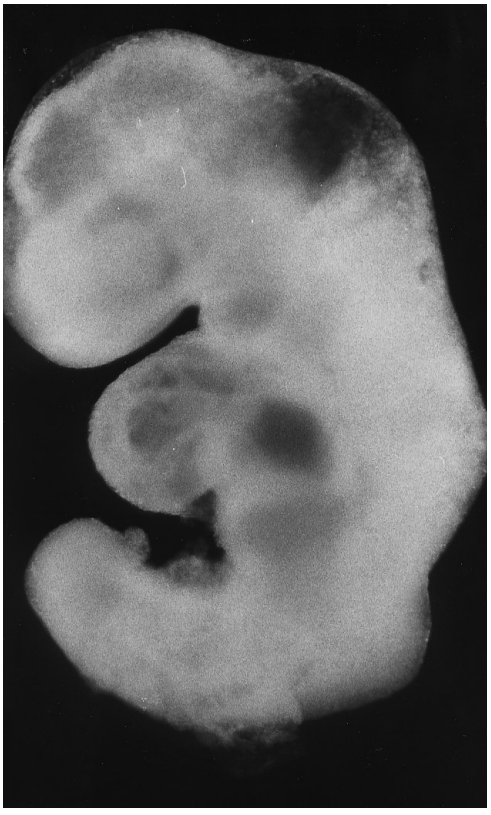

**Fig. 2.** Rat embryo after 48 h culture in vitro, exposed to 30 mmol/l glucose in 75 MD serum, showing multiple severe abnormalities; such as open neural tube, retarded trunk and tail regions, enlarged heart, a crown-rump length of 2.6 mm, but only 12 somites (malformation score: 10)

of either 10 or 30 mmol/l, with the exception of N 10G embryos, which consumed half the amount of glucose in the medium during the second 24 h of culture, thus ending the culture period with a glucose concentration of 4.5 mmol/l (Table 2). The fructosamine concentration varied considerably between the different culture conditions, but showed a tendency to increase in the 75 MD culture tubes. Most of the protein concentrations were found to be between 50 and 65 mmol/l, with no discernible pattern with respect to diabetes or serum additions.

The lipid-associated metabolites showed very different concentration changes. Cholesterol concentrations were constant between the different culture conditions, whereas triglyceride levels were tripled (from around 1.5 mmol/l in the N cultures to around 4.5 mmol/l in the MD cultures) with no difference between 50 MD and 75 MD media. A similar pattern was seen for β-hydroxybutyrate, whose values were almost 10 times higher in the MD cultures compared to N cultures, again with no difference between the 50 MD and 75 MD media. In contrast, creatinine was increased only in the 75 MD samples compared to the other two serum types (N and 50 MD), which did not differ within themselves. The GOT and GPT

**Table 1.** Concentrations of folate, vitamin $B_{12}$, as well as amino acids in the serum from normal rats (N), and rats rendered manifestly diabetic by a single injection of either 50 mg/kg STZ (50 MD) or 75 mg/kg STZ (75 MD)

| Serum metabolite | | N | 50 MD | 75 MD |
|---|---|---|---|---|
| Folate | nmol/l | 49 ± 1 | 31 ± 1 | 48 ± 1 |
| Vitamin $B_{12}$ | pmol/l | 701 ± 5 | 722 ± 23 | 886 ± 55 |
| Taurine | μmol/l | 291 ± 4 | 241 ± 13 | 818 ± 20[ab] |
| Aspartic acid | μmol/l | 46 ± 1 | 39 ± 3 | 93 ± 8[ab] |
| Threonine | μmol/l | 254 ± 4 | 183 ± 7[a] | 342 ± 10[ab] |
| Serine | μmol/l | 258 ± 1 | 175 ± 5[a] | 269 ± 8[b] |
| Asparagine | μmol/l | 38 ± 11 | 26 ± 1 | 72 ± 5[ab] |
| Glutamic acid | μmol/l | 134 ± 1 | 70 ± 2[a] | 205 ± 7[ab] |
| Glutamine | μmol/l | 597 ± 1 | 378 ± 12[a] | 943 ± 30[ab] |
| Proline | μmol/l | 258 ± 4 | 144 ± 5[a] | 174 ± 28[a] |
| Glycine | μmol/l | 314 ± 1 | 215 ± 8[a] | 443 ± 15[ab] |
| Alanine | μmol/l | 562 ± 4 | 243 ± 10[a] | 611 ± 18[b] |
| Citrulline | μmol/l | 90 ± 1 | 58 ± 3[a] | 83 ± 0.3[ab] |
| Valine | μmol/l | 198 ± 2 | 332 ± 13[a] | 640 ± 16[ab] |
| Methionine | μmol/l | 63 ± 1 | 45 ± 1[a] | 69 ± 2[b] |
| Isoleucine | μmol/l | 115 ± 1 | 181 ± 7[a] | 307 ± 8[ab] |
| Leucine | μmol/l | 155 ± 2 | 257 ± 9[a] | 504 ± 13[ab] |
| Tyrosine | μmol/l | 92 ± 2 | 54 ± 5[a] | 98 ± 0.3[b] |
| Phenylalanine | μmol/l | 78 ± 3 | 56 ± 2[a] | 99 ± 4[ab] |
| Ornithine | μmol/l | 219 ± 1 | 51 ± 2[a] | 126 ± 4[ab] |
| Lysine | μmol/l | 378 ± 3 | 211 ± 8[a] | 405 ± 12[b] |
| Histidine | μmol/l | 60 ± 2 | 53 ± 2 | 169 ± 5[ab] |
| Arginine | μmol/l | 70 ± 3 | 107 ± 3[a] | 173 ± 8[ab] |

[a] $p < 0.05$ vs N, [b] $p < 0.05$ vs 50 MD (ANOVA)

activities did not differ between the culture conditions. Treatment with superoxide dismutase and N-acetylcysteine did not affect the metabolite concentrations in any consistent manner (Table 2).

The outcome of the embryo cultures is shown in Table 3 and Figure 3. In N 10G culture, the embryos showed high protein and DNA content, a somite number of 30, a crown-rump length of 4 mm and a malformation score of 0.3, indicating a virtually undisturbed embryonic development (Fig. 3 left column). Increasing the glucose concentration to 30 mmol/l had profound effects. We found decreased protein and DNA content, decreased somite number and crown-rump length, and a large increase in malformation score (Table 3), mainly because of an increased number of severe malformations (Fig. 3). Culture in 50 MD medium (at 30 mmol/l glucose) resulted in further decreased protein content, somite number, and crown-rump length (compared to N 30G culture), whereas DNA content, protein/DNA ratio, and malformation score were disturbed to the same degree in the N 30G and 50 MD 30G cultures (Table 3). Culture in 75 MD medium, however, yielded further decreased protein content, somite number, as well as crown-rump length (compared with 50 MD culture). Also, the DNA content and the malformation score of these embryos were different from those of the N 30G embryos (Table 3 and Fig. 2).

Treatment with SOD and NAC slightly increased protein/DNA ratio, and decreased the

**Table 2.** Concentrations of metabolites and enzyme activities in pooled media (from 3 culture tubes) after 24 h and 48 h culture in vitro in serum from normal (N), and manifestly diabetic rats

| Serum metabolite | N10G | N30G | N30G +S | N30G +N | 50MD30G | 50MD30G +S | 50MD30G +N | 75MD30G | 75MD30G +S | 75MD30G +N |
|---|---|---|---|---|---|---|---|---|---|---|
| *After 24 h culture* | | | | | | | | | | |
| Glucose (mmol/l) | 10.2 | 30.8 | 30.1 | 29.0 | 31.4 | 29.9 | 29.4 | 30.4 | 32.6 | 29.8 |
| Fructosamine (μmol/l) | 91 | 117 | 82 | 107 | 111 | 116 | 153 | 156 | 110 | 146 |
| Protein (mmol/l) | 69.3 | 56.3 | 55.6 | 57.6 | 53.3 | 51.2 | 58.3 | 49.0 | 51.0 | 53.5 |
| Cholesterol (mmol/l) | 2.53 | 1.49 | 1.35 | 1.73 | 1.54 | 1.49 | 1.65 | 1.18 | 1.39 | 1.69 |
| Triglycerides (mmol/l) | 2.03 | 1.40 | 1.33 | 1.85 | 5.86 | 6.13 | 4.66 | 4.27 | 4.66 | 5.29 |
| $\beta$-HB (mmol/l) | 0.78 | 0.61 | 0.60 | 0.59 | 7.71 | 8.35 | 6.11 | 7.84 | 6.94 | 14.78 |
| Creatinine (μmol/l) | 69 | 49 | 50 | 49 | 62 | 61 | 59 | 118 | 132 | 103 |
| GOT (μKat/l) | 1.43 | 0.86 | 1.10 | 0.91 | 1.44 | 1.15 | 1.98 | 1.21 | 1.27 | 1.46 |
| GPT (μKat/l) | 0.46 | 0.43 | 0.16 | 0.35 | 0.68 | 0.54 | 0.92 | 0.18 | 0.49 | 0.56 |
| *After 48 h culture* | | | | | | | | | | |
| Glucose (mmol/l) | 4.5 | 29.2 | 30.5 | 30.7 | 30.7 | 30.5 | 29.8 | 31.3 | 33.7 | 29.1 |
| Fructosamine (μmol/l) | 84 | 105 | 92 | 152 | 150 | 123 | 217 | 152 | 125 | 158 |
| Protein (mmol/l) | 68.5 | 65.6 | 69.5 | 91.8 | 57.5 | 61.8 | 83.2 | 59.4 | 59.2 | 62.2 |
| Cholesterol (mmol/l) | 1.88 | 1.42 | 1.72 | 3.43 | 1.65 | 1.70 | 2.80 | 1.65 | 1.53 | 1.87 |
| Triglycerides (mmol/l) | 1.90 | 1.73 | 1.65 | 3.16 | 6.16 | 7.16 | 6.29 | 5.63 | 5.52 | 6.06 |
| $\beta$-HB (mmol/l) | 0.89 | 1.00 | 1.04 | 0.97 | 8.47 | 10.02 | 7.24 | 8.45 | 8.34 | 8.37 |
| Creatinine (μmol/l) | 59 | 61 | 62 | 85 | 67 | 71 | 91 | 138 | 146 | 87 |
| GOT (μKat/l) | 0.96 | 0.94 | 1.19 | 1.52 | 1.27 | 1.31 | 3.19 | 1.38 | 1.54 | 1.82 |
| GPT (μKat/l) | 0.46 | 0.49 | 0.26 | 0.56 | 0.59 | 0.48 | 1.39 | 0.67 | 0.43 | 0.63 |

Cultures performed in 10 mmol/l glucose (10G) or 30 mmol/l glucose (30G) with addition of superoxide dismutase (+S) or N-acetylcysteine (+N) as shown. β-HB denotes β-hydroxybutyrate

**Table 3.** Outcome of embryo culture in serum from normal (N) or manifestly diabetic rats

| Type of serum | *n* | Protein content (μg/embryo) | DNA content (μg/embryo) | Protein/DNA ratio | Somites | Crown-rump length (mm) | Malformation score |
|---|---|---|---|---|---|---|---|
| N 10G | 19 | 306 ± 10 | 55 ± 2 | 5.6 ± 0.1 | 30.6 ± 0.4 | 4.09 ± 0.08 | 0.3 |
| N 10G+S | 20 | 301 ± 11 | 49 ± 2 | 6.2 ± 0.3[a] | 28.7 ± 0.2 | 3.68 ± 0.04[a] | 0.1 |
| N 10G+N | 22 | 311 ± 12 | 49 ± 1 | 6.3 ± 0.3[a] | 28.6 ± 0.4 | 3.63 ± 0.07[a] | 0.1 |
| N 30G | 24 | 269 ± 15[a] | 43 ± 3[a] | 6.3 ± 0.2 | 23.2 ± 1.3[a] | 3.60 ± 0.10[a] | 6.9[a] |
| N 30G+S | 19 | 306 ± 16[b] | 47 ± 2[a] | 6.5 ± 0.2 | 29.1 ± 0.5[b] | 3.88 ± 0.08 | 1.1[b] |
| N 30G+N | 20 | 280 ± 14 | 46 ± 3[a] | 6.3 ± 0.3 | 27.6 ± 0.8[ab] | 3.62 ± 0.08[a] | 2.1[b] |
| 50MD30G | 24 | 216 ± 12[ab] | 38 ± 2[a] | 6.0 ± 0.4 | 19.8 ± 1.2[ab] | 3.21 ± 0.09[ab] | 7.3[a] |
| 50MD30G+S | 19 | 214 ± 11[ab] | 36 ± 2[ab] | 6.0 ± 0.2 | 25.2 ± 1.1[ac] | 3.46 ± 0.07[a] | 3.3[abc] |
| 50MD30G+N | 20 | 266 ± 10[ac] | 55 ± 2[bc] | 4.9 ± 0.1[bc] | 27.0 ± 1.0[abc] | 3.67 ± 0.07[ac] | 2.5[bc] |
| 75MD30G | 29 | 136 ± 10[abc] | 22 ± 2[abc] | 7.1 ± 0.6[ac] | 10.0 ± 0.9[abc] | 2.50 ± 0.11[abc] | 10.0[ab] |
| 75MD30G+S | 19 | 128 ± 13[abc] | 20 ± 2[abc] | 6.2 ± 0.3[d] | 17.5 ± 1.2[abd] | 2.66 ± 0.12[abc] | 8.7[a] |
| 75MD30G+N | 20 | 147 ± 6[abc] | 30 ± 2[abcd] | 5.2 ± 0.3[bd] | 16.6 ± 1.0[abcd] | 2.69 ± 0.07[abc] | 9.8[ab] |

[a] $p < 0.05$ vs N 10G, [b] $p < 0.05$ vs N 30G, [c] $p < 0.05$ vs 50MD30G, [d] $p < 0.05$ vs 75MD30G (ANOVA or chi square statistics [with Yates' correction])

Manifestly diabetic (MD) rats were made diabetic with i.v. STZ at 50 mg/kg body weight (50 MD) or 75 mg/kg (75 MD). Cultures performed in 10 mmol/l glucose (10 G) or 30 mmol/l glucose (30 G) with addition of superoxide dismutase (+S) or N-acetylcystein (+N) as shown

crown-rump length in the N 10G embryos, whereas there was no effect on malformation score in this group (Table 3 and Fig. 3). In the embryos cultured at 30 mmol/l glucose in N serum, the addition of SOD and NAC improved both somite number and malformation score, whereas protein content was increased by SOD only, and the other parameters were not significantly affected by the antioxidant treatment.

In the embryos cultured at 30 mmol/l glucose in 50 MD serum, there was, again, a distinct improvement in the somite number and malformation score by the antioxidant treatment, whereas protein content, DNA content, protein/DNA ratio, and crown-rump length were improved by NAC only. The embryos cultured in 75 MD serum were helped by the antioxidants with regard to somite numbers and protein/DNA ratio, whereas no effect on malformation score

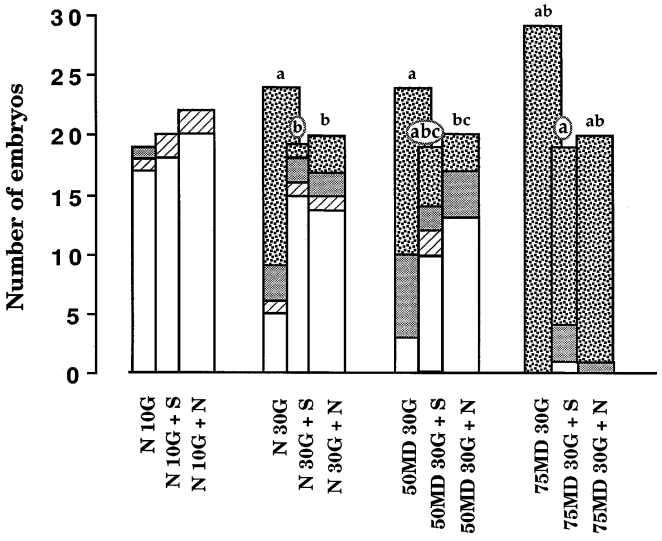


**Fig. 3.** Morphological outcome of culture in serum from normal (N), and manifestly diabetic rats, the latter were given an i.v. injection of STZ, either at 50 mg/kg body weight (50 MD) or at 75 mg/kg body weight (75 MD). The cultures were performed either in 10 mmol/l glucose (10G) or in 30 mmol/l glucose concentration (30 G), with addition of superoxide dismutase (+S) or N-acetylcysteine (+N). The embryos were scored as normal (□), minor malformation (▨), slight malformation (▩), or severe malformation (▦). [a]$p < 0.05$ vs N 10G, [b]$p < 0.05$ vs N 30G, [c]p < 0.05 vs 50MD 30G, [d]$p < 0.05$ vs 75MD 30G (chi square statistics with Yates' correction)

was seen (Table 3 and Fig. 3). Furthermore, only NAC increased DNA content in the 75 MD embryos.

## Discussion

The main result of the present study was the demonstration of a teratogenic potential of diabetic serum, in addition to the teratogenicity provided by increased glucose and β-hydroxybutyrate concentrations, and that supplementation of the antioxidants SOD and NAC to the teratogenic culture medium protected the embryos from dysmorphogenesis. These findings corroborate earlier claims of the multifactorial nature of the teratogenicity of diabetic pregnancy [4, 9], and supports the notion of an excess of free oxygen radical activity in the embryos as an integral part of the teratogenic process [10–12].

The teratogenic potential of the different serum milieus was found to be markedly varied. Thus, increasing the glucose concentration from 10 to 30 mmol/l in normal serum caused a clear reduction in the size of the embryos, as well as an increased incidence of somatic malformations, thereby illustrating the teratogenic effect of an increased glucose concentration *per se*. On the other hand, culturing embryos in the same glucose concentration (30 mmol/l) in diabetic (50 MD) serum yielded further decreased embryonic size compared with culture in normal serum. This finding indicates the presence of teratogenic components in diabetic serum other than the high glucose concentration [7, 8].

In the 50 MD serum, folate concentration was decreased, whereas the branched chain amino acids, triglyceride and β-hydroxybutyrate concentrations were increased in comparison with N serum, all of which have been previously associated with disturbed embryonic development [4, 6, 11, 30, 31]. In addition, there were pronounced changes in a number of amino acid concentrations, the significance of which remain to be determined [30, 32].

The teratogenic factors in diabetic serum are only partly known. Thus, increased glucose and increased β-hydroxybutyrate concentrations have been shown to induce embryonic dysmorphogenesis in vitro by themselves [5, 6], and should also be able to do so in a diabetic serum environment. The same argument should apply to branched chain amino acids, since α-ketoisocaproic acid (transaminated metabolite of leucine) is able to induce embryonic dysmorphogenesis in vitro [11], but other components are less well characterized. Indirect evidence of a teratogenic effect of increased glucose, β-hydroxybutyrate, branched chain amino acids, and triglyceride concentration was obtained in vivo by multiple linear regression analysis of maternal diabetic serum [4], whereas the importance of folate for in vitro embryonic development has been shown in studies with dialysed serum in culture of embryos [30]. In the present study, the marked simultaneous changes in these factors may, therefore, explain the differences in teratogenicity between N and 50 MD serum.

When SOD and NAC were added to the N and 50 MD serum cultures we found marked protection from the teratogenic effects of the high glucose and diabetic serum milieu, and a clear, but less complete protection against the growth retardation caused by the increased glucose (N) and diabetic environment (50 MD). The exact nature of the teratogenic process is not known, but it may be associated with a direct or relative excess of free oxygen radicals in the embryo, since SOD would lower the embryonic levels of superoxide, and NAC increases the cellular levels of intracellular antioxidant (reduced) glutathione (GSH) by providing the rate-limiting amino acid, cysteine, for the synthesis of GSH. Indeed, in previous experiments we and others have found evidence for this notion. In vitro, culture of embryos with the antioxidative enzymes SOD, catalase and glutathione peroxidase blocked the embryonic dysmorphogenesis caused by the high glucose concentration [10], and SOD protected against increased β-hydroxybutyrate and α-ketoisocaproate concentration [11]. Embryos transgenic for the CuZnSOD gene and exhibiting increased SOD activity, were found to be partly shielded from the teratogenic effects of high glucose and

β-hydroxybutyrate in vitro [33], as well as maternal diabetes in vivo [34]. Furthermore, we have shown that supplement of the antioxidant butylated hydroxytoluene in the diet to diabetic rats protects from congenital malformations [35], and we [36] and others [37] have presented evidence of an antiteratogenic effect of vitamin E treatment of pregnant diabetic rats.

It is now established that antioxidative therapy blocks diabetes-induced embryonic dysmorphogenesis in vivo and in vitro. The exact mechanism for this protection is not yet completely elucidated. A diabetic state in vivo, as well as high glucose concentration in vitro induce SOD activity in the embryo [10], in particular mitochondrion-specific MnSOD expression is increased [38]. This is interesting in view of the demonstrated morphological [12] and functional [39] changes of the mitochondria in neural tissue of rat embryos exposed to high glucose or maternal diabetes, suggesting that these organelles may have a role in a possible endogenous overproduction of free oxygen radicals as a conceivable consequence of increased availability of oxidative substrates. This notion is supported by the demonstration of inhibition of glucose- and pyruvate-induced teratogenicity in vitro by supplementation of the inhibitor α-cyano-hydroxycinnamic acid, which hampers mitochondrial uptake of pyruvate [11].

On the other hand, direct demonstration of 2′-7′-dichlorofluorescein diacetatereactive oxygen radicals, i. e. hydrogen peroxide and hydroxyl radicals, was not possible in isolated cells from embryos of normal and diabetic rat pregnancies exposed to high glucose and high β-hydroxybutyrate concentrations in vitro at the end of the teratogenic period [31, 40]. This may illustrate a difference in acute and chronic effects on the embryonic tissue exerted by a diabetic environment, but could also indicate that the major effect of the diabetic milieu is a decrease in antioxidant capacity rather than an outright increase in oxygen radical production. This notion is strengthened by the clear antiteratogenic effect of supplementing either NAC (present study) or GSH ester [41] to high-glucose cultured embryos. In addition, it has been shown that culture of rat embryos in high glucose concentration decreases cellular glutathione levels and decreases the activity of the rate-limiting GSH-synthesising enzyme, γ-glutamylcysteine synthetase [41].

In this context the possibility of varied oxidative responses in different cell populations should be considered. Thus, in developing cranial neural crest cells, increased glucose concentration decreases migration and proliferation in vitro, whereas somitic cells do not respond at all to this challenge [42]. Furthermore, addition of NAC to the culture medium normalizes neural crest cell development, indicating that the retardation of the neural crest cells contains a radical element [42].

Embryos cultured in the diabetic serum from the rats given the high STZ dose showed a slightly different result from those of the N serum and low-dose STZ sera. We found that almost all embryos, cultured in serum from 75 MD donors were severely malformed, and that we could not prevent or decrease the malformation rate with addition of SOD or NAC, which was possible with 50 MD serum. The embryos were more affected than embryos cultured under the other two conditions, and, furthermore the alleviation of the growth retardation offered by the two antioxidants was marginal, and mainly affected the somite number. Also, we did not find a dose-response effect on the metabolite concentrations with respect to STZ dose which suggests that some factor other than the severity of diabetes affects the changes in embryonic outcome.

STZ treatment caused increased triglyceride and β-hydroxybutyrate concentrations in both 50 MD and 75 MD serum, and a selective increase in creatinine concentration in the 75 MD serum (similar trend for fructosamine). These findings suggest that some other change in addition to increased severity of the diabetic state has occurred in the 75 MD rats, and, consequently, affected the growth-promoting properties of the serum of these rats but this needs further investigation.

In conclusion, we have shown that diabetic serum has a teratogenic effect on embryonic development, a capacity residing in the alteration of several serum components in addition to glucose. Improving the embryonic capability to scavenge oxygen radicals, either by increasing SOD activity or by supplying a rate-limiting precursor (NAC) for the enhanced synthesis of GSH, blocks the embryonic dysmorphogenesis.

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