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Detection of Teratogens in Human Serum Using Rat Embryo Culture: Cancer and Epilepsy Treatments

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Abstract: Growth (protein and DNA contents) of headfold stage rat embryos cultured for 48 hours on human serum was enhanced by glucose supplementation. Embryo growth varied with the source of the serum. Sera from 3 of the 19 control subjects produced abnormal embryos. Sera from 5 subjects undergoing cancer chemotherapy and 6 subjects receiving anticonvulsants were either lethal or teratogenic to cultured rat embryos.

Advances in procedures for in vitro culture of rat embryos during stages of organogenesis have provided a model system in which to study teratology (1). We recently showed that such cultures could be used to monitor changes in serum teratogenicity with time when sera from rats injected with cadmium chloride or cyclophosphamide were used as culture media (2). We report here on the extension of this approach to the detection of potential teratogens in human serum.

Culture of headfold stage rat embryos on serum other than from rats has not previously been successful (3). However, it has been reported that somite stage rat embryos could be cultured for 18–22 hours on human serum (4). Studies in this laboratory and by others (5) using headfold stage rat embryos and a 48 hour culture period indicated that human serum was inferior to rat serum in supporting embryo growth and development. Estimates of glucose consumption by cultured embryos (6) and the higher level of glucose in rat serum (1.8 mg/ml) as compared to human serum (0.98 mg/ml) suggested that the low glucose content of human serum might be limiting embryo growth. When 2.0 to 3.0 mg of glucose were added per ml of human serum (final glucose concentration of approximately 3.0 to 4.0 mg/ml), the accumulation of protein by headfold stage rat embryos after 48 hours of culture increased by approximately 3 fold and the accumulation of DNA by approximately 5 fold over those embryos cultured on unsupplemented human serum (Fig. 1, 2a and 2b). In subsequent experiments all serum samples were analyzed for glucose concentration by the glucose oxidase method (7) and were adjusted to a final concentration of 3 mg/ml. In addition to the glucose supplement, the preparation of human serum for use as a culture medium involved the same procedures that have been used for rat serum including immediate centrifugation after withdrawal (8), heat inactivation for 30 minutes at 56°C (8),

sterile filtration through HA type Millipore filter, supplementation with (mg/ml) 0.66 streptomycin sulfate and 0.0006 penicillin-G-potassium and dilution with 10 per cent by volume sterile water (2). Glucose solution used as a supplement replaced a comparable volume of water used in dilution.

An estimate of variation between individuals was made by culturing embryos on serum samples from 10 male subjects ages 22 to 33 and 9 female subjects ages 20 to 25. They were considered to be in good health, were non-smokers and were not taking medication including oral contraceptives. Variations in the protein and DNA contents of embryos were observed among the serum samples, but the average for males did not differ significantly from that for females (Table 1). In comparison to previously published data from this laboratory (2) the embryo protein content on human serum was 73 per cent and the DNA 117 per cent of that obtained by embryos cultured on rat serum. Histological comparison between embryos of similar size cultured on rat serum and on human serum supplemented with glucose showed comparable morphological development (for example, compare Fig. 2c and 2d). The embryos had closed neural tubes, approximately 25 somite pairs, forelimb buds, otocysts and 3 pairs of both aortic arches and branchial pouches. Embryos cultured on control serum which were abnormal (Table 1) generally contained less protein and DNA than the other controls and showed failure of the neural tube to close at various levels (exencephaly) and incomplete body curvature. The frequency of abnormalities was greater with female serum (29 per cent) than with male serum (10 per cent) but most of the abnormalities involved serum from only two females and one male.

To test the ability of cultured rat embryos to detect teratogens in human serum, subjects were selected who were receiving continuous drug treatment. Sera from cancer chemotherapy subjects 20, 21 and 23 were lethal

to embryos during the first 24 hours of culture (Table 2). When these serum samples were diluted 50 per cent by volume with control human serum they remained embryo lethal. Serum from subject 23 was also tested at a dilution of 1:3 by volume with control human serum and found to be lethal. Sera from subjects 24 (cyclophosphamide) and 25 (Tamoxifen) allowed embryo survival for 48 hours but all embryos were abnormal (Table 2) and protein to DNA ratios were higher than controls (controls ranged approximately 6 to 11 while the ratio for subject 24 was 15 and for subject 25 the ratio was 19). With serum from subject 24, body curvature and limb bud formation were less advanced than controls (Fig. 2e) while histological examination (Fig. 2f) revealed exencephaly extending from the anterior tip of the forebrain (telencephalon) to the level of the otic pit in the hindbrain (myelencephalon), dilation of the internal carotid artery, retarded development of the eye and reduced numbers of somites (16 pairs), aortic arches (1 pair) and branchial pouches (2 pairs). Serum from subject 25 caused exencephaly from the forebrain (telencephalon) through the midbrain (mesencephalon), reduction in somite number (18 pairs) and absence of forelimb buds.

In one subject, 21, it was possible to obtain sera samples not only during chemotherapy but also 3 months after the cessation of chemotherapy. Serum obtained during treatment was embryo lethal whereas serum obtained post-treatment supported embryo protein and DNA accumulations comparable to controls (Table 2). Gross observations indicated that morphological parameters were also comparable to controls. When serum from subject 22 was used as a culture medium embryos similar to controls were also obtained. This subject had been diagnosed as having cancer but had not as yet received chemotherapy.

All embryos cultured on serum from the six subjects receiving

anticonvulsants survived 48 hours of culture. With four of the six serum samples, the protein and DNA values for the embryos did not differ significantly from controls (Table 3). Although embryos cultured on serum from subject 28 were extremely small and showed extensive tissue necrosis, all other embryos appeared morphologically comparable to controls (Fig. 2g) in several aspects including normal body curvature, presence of forelimb buds, development of the pharyngeal region and nasal placodes. Somite numbers ranged slightly lower than controls: 17 to 24 pairs as compared to 21 to 26 pairs. In spite of the similarities to controls, all embryos without exception were exencephalic and failed to develop optic vesicles. The extent to which the neural tube was open and the regions involved varied with the subjects but were consistent for each serum sample. For example, with subject 27 the exencephaly extended from the middle of the forebrain (telencephalon and diencephalon) through the hindbrain (myelencephalon) (Fig. 2f). With subject 29 the brain was open from the anterior tip of the forebrain (telencephalon) to the level of the midbrain (mesencephalon).

In this report we have demonstrated that rat embryos can be cultured on human serum when supplemented with glucose and that they can be used to detect potential teratogens in human serum. Serum samples from subjects receiving drugs that have been shown to be teratogenic in animals (9) or that have been implicated as human teratogens (10) were either embryo lethal or teratogenic in all cases. However, at least three of the nineteen control sera samples were also detrimental to cultured rat embryos. In this regard we have observed embryo lethality as well as abnormalities with sera from several subjects suffering from colds and with rat serum obtained from animals suspected of having pneumonia. Clearly, extensive prospective studies would be required to establish a possible relationship between the affects

of human serum on cultured rat embryos and the occurrence of developmental abnormalities in man.

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Figure Legends

Figure 1: Protein and DNA contents of headfold stage rat embryos cultured for 48 hours on human serum supplemented with various amounts of glucose. Subject 1 was used for male serum while equal amounts of subjects 12 and 16 were combined for female serum. Nulliparous CD strain females (Charles River Breeding Laboratories, Inc., Willmington, Massachusetts), mated with CD strain males, were sacrificed for embryos at 9.5 days gestation (the morning of positive vaginal smear for sperm was considered 0.5 days). Headfold stage embryos were selected and cultured with intact yolk-sacs and ectoplacental cones but Reichert's membranes were removed (2). Culture vessels (11) containing 3 embryos per 2 ml of media were maintained at 37.5°C, rotated at 30 rpm and gassed with 5% O_2/5% CO_2/90% N_2 at zero time and 12 hours, 20% O_2/5% CO_2/75% N_2 at 24 hours and 40% O_2/5% CO_2/55% N_2 at 36 hours (2). For protein and DNA determinations embryos were separated from extraembryonic membranes, rinsed in saline, placed overnight in 5% trichloroacetic acid and homogenized. After centrifugations the pellets were extracted with 95% ethanol, 1:3 chloroform ethanol for 15 minutes at 70°C and 95% ethanol. DNA was then extracted with 5% perchloric acid at 70°C for 20 minutes (12). The remaining protein precipitate was rinsed with 95% ethanol and dissolved in NaOH. Diphenylamine was used for DNA quantitation (13) and the method of Lowry et al (14) for protein.

Figure 2: Headfold stage rat embryos after 48 hours of culture on various sera. Live embryos were either photographed immediately after removal of extraembryonic membranes or representative embryos with intact extraembryonic membranes were fixed immediately in Carnoy's. Following routine processing, paraffin sections were cut at 7 μm and stained with Ehrlich's hematoxylin and PAS. Whole embryos were photographed at 25X and sections at 60X. (a) Human serum without added glucose. (b) Human serum with 3.0 mg/ml glucose added.

(c) Rat serum. (d) Human serum adjusted to 3.0 mg/ml glucose. (e and f) Serum from subject 24 undergoing cancer chemotherapy. (g and h) Serum from subject 27 receiving anticonvulsants. Di, diencephalon; My, myelencephalon; OV, optic vesicle; OS, optic sulcus; NP, nasal placode; IC, internal carotid artery.

Table 1: Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum.

Table 2: Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum from subjects undergoing cancer chemotherapy. Treatments for each subject were as follows with the medication received on the day of sampling in parentheses: 20, vincristine, 1 mg iv/3 weeks, CCNU, 100 mg po/6 weeks (vincristine); 21, vincristine, 2 mg iv/ 3 weeks, adriamycin, 30 mg iv/3 weeks, CCNU, 100 mg po/6 weeks, cyclophosphamide, 1 g po/6 weeks (vincristine and adriamycin); 23, cyclophosphamide, 100 mg po/day; 24, cyclophosphamide, 100 mg po/day, methotrexate, 75 mg iv/4 weeks, 5-fluorouracil, 1.5 g iv/4 weeks (methotrexate and 5-fluorouracil); 25, Tamoxifen, 20 mg po/day.

Table 3: Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum from subjects receiving anticonvulsants. Daily oral medications for each subject were as follows: 26, tegretol, 400 mg; 27, peganone, 500 mg, tegretol, 1.2 g, primidone, 875 mg, depakene, 1.25 g; 28, primidone, 750 mg, carbamezepine, 800 mg, depakene, 1.5 g; 29, tegretol, 800 mg, mellaril, 250 mg, Dilantin, 350 mg; 30, tegretol, 800 mg, depakene, 1 g, primidone, 500 mg; 31, Dilantin, 300 mg.

TABLE 1

Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum.

SUBJECT	NO. EMBRYOS	NO. ABNORMAL	PROTEIN (μg/embryo ± S.E.)	DNA (μg/embryo ± S.E.)
			MALE	
1	5	0	117.3 ± 5.3	10.9 ± 0.9
2	6	0	92.4 ± 6.4	11.9 ± 0.4
3	6	0	92.3 ± 6.4	12.4 ± 1.0
4	6	0	85.8 ± 4.8	11.5 ± 0.7
5	6	0	85.2 ± 7.4	10.1 ± 1.2
6	6	1	81.0 ± 8.1	8.7 ± 1.0
7	6	0	74.1 ± 8.2	11.6 ± 1.7
8	6	0	70.7 ± 6.5	8.0 ± 0.8
9	6	0	53.3 ± 4.9	7.8 ± 1.0
10	6	5	42.8 ± 3.2	5.8 ± 0.4
N = 10	59	6	79.5 ± 6.6	9.9 ± 0.7
			FEMALE	
11	3	0	100.0 ± 10.2	13.7 ± 0.6
12	3	0	94.8 ± 1.7	12.1 ± 1.0
13	6	1	83.0 ± 4.0	10.9 ± 0.6
14	6	1	75.2 ± 8.1	7.7 ± 0.8
15	6	0	66.1 ± 4.6	8.7 ± 0.9
16	6	0	64.5 ± 7.6	9.3 ± 0.7
17	6	6	62.9 ± 6.4	8.2 ± 0.5
18	6	6	57.9 ± 7.3	9.3 ± 1.2
19	6	0	48.0 ± 5.7	8.0 ± 1.0
N = 9	48	14	72.5 ± 5.8	9.8 ± 0.7
			OVERALL AVERAGE	
N = 19	118	20	76.2 ± 4.4	9.8 ± 0.5

TABLE 2

Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum from subjects undergoing cancer chemotherapy.

SUBJECT	NO. EMBRYOS	NO. ABNORMAL	PROTEIN (μg/embryo ± S.E.)[a]	DNA (μg/embryo ± S.E.)[a]
			MALE	
20	3	lethal	10.5**	0.7**
21	3	lethal	9.1**	0.6**
21 (Post-Treat.)	3	0	81.7 ± 2.9	10.5 ± 0.6
22 (No Treat.)	3	0	86.6 ± 9.1	7.7 ± 0.6*
			FEMALE	
23	3	lethal	20.6**	0.7**
24	3	3	54.4 ± 1.7*	3.5 ± 0.6**
25	3	3	88.5 ± 2.0	4.6 ± 1.0**

[a] The level of significance of the difference between experimental values and male or female control values by Student's T test: *$p < 0.05$; **$p < 0.01$.

TABLE 3

Protein and DNA contents and abnormality frequencies of headfold stage rat embryos cultured for 48 hours on human serum from subjects receiving anticonvulsants.

SUBJECT	NO. EMBRYOS	NO. ABNORMAL	PROTEIN (μg/embryo ± S.E.)[a]	DNA (μg/embryo ± S.E.)[a]
			MALE	
26	3	3	63.8 ± 4.8	8.6 ± 0.4
27	3	3	60.8 ± 4.0*	5.9 ± 1.1*
28	3	3	13.8**	4.2**
29	3	3	83.0 ± 1.3	9.6 ± 0.4
30	3	3	79.3 ± 6.6	8.6 ± 0.4
			FEMALE	
31	3	3	71.9 ± 1.2	8.9 ± 0.4

[a] The level of significance of the difference between experimental values and male or female control values by Student's T test: *$p < 0.05$; **$p < 0.01$.

Fig 1

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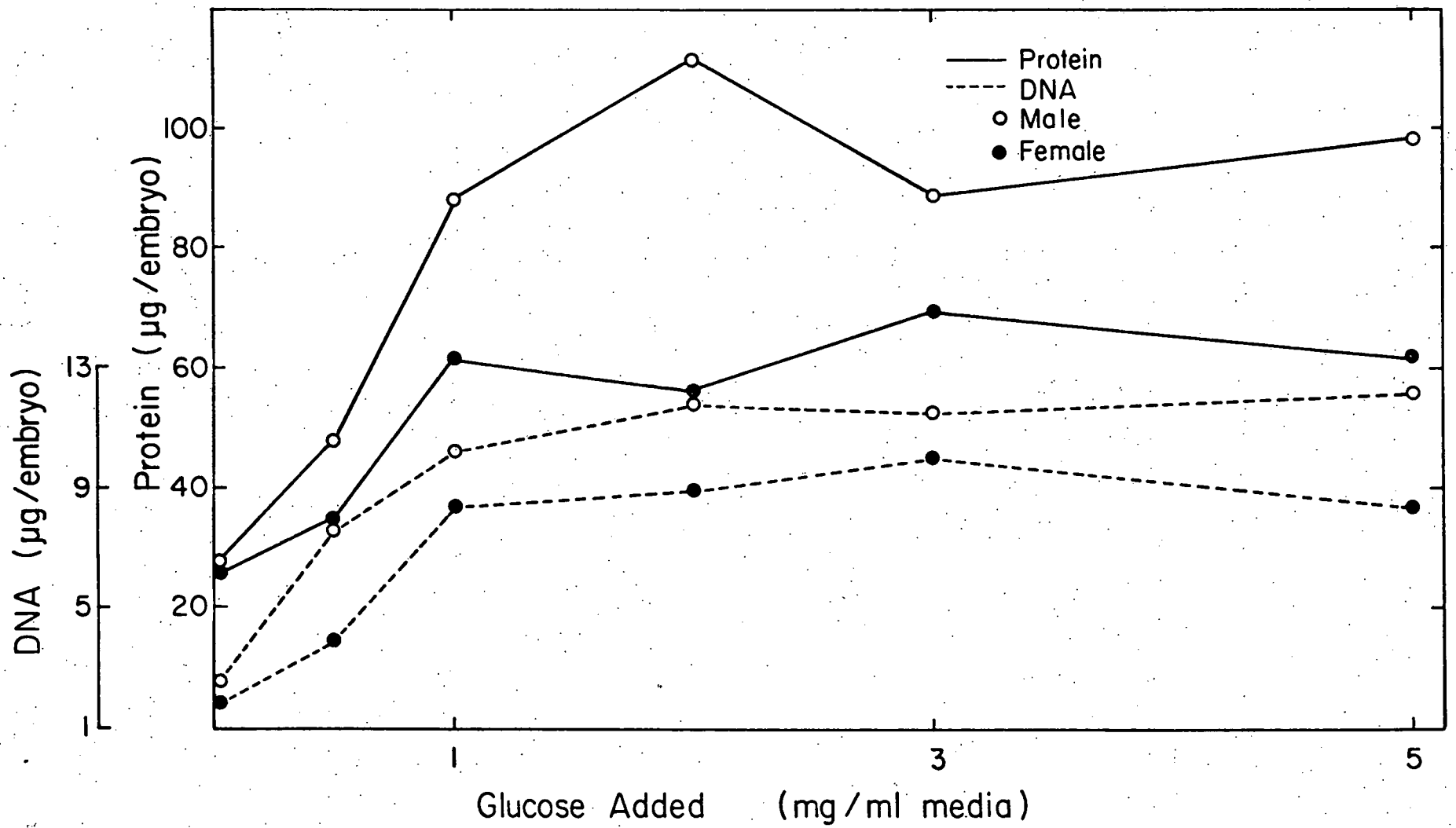

Fig 1