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The development of the cerebral mantle in the mouse embryo after brief *in utero* hyperthermic stress

M. Tayo Shokunbi and A.B.O. Desalu
Department of Anatomy, University of Ibadan, Ibadan, Nigeria.

Summary

The width of the different layers of the cerebral mantle of 16-day mouse embryos was measured after exposure of pregnant dams to a hyperthermic stress of 43°C for 10 minutes in a laboratory oven, during the period of neural tube closure. The dams were exposed on day $7\frac{1}{2}$ or $8\frac{1}{2}$ post coitum. A control group of mice was placed in the oven at ambient temperature (28°C) for 10 minutes.

At 43°C oven temperature, the core temperature of the pregnant dams was elevated by 2-3°C ($P \leq 0.001$). In non-pregnant mice, this degree of heat stress results in a sustained hyperthermia of about six minutes and a return to normal temperature 15 minutes after removal from the oven. There were no congenital malformations of the central nervous system. The mean litter sizes, percentage resorptions and fixed embryonic weights were similar in the experimental and control groups.

The width of the cortical plate was significantly reduced in all the regions measured ($P \leq 0.001$). The width of the matrix layer was reduced only in the fronto-parietal region.

The results suggest that this sub-teratogenic dose of hyperthermic stress reduces neuronal population in the cerebral cortex. This may be the structural substrate for the impairment of function in animals heat stressed during prenatal development.

Résumé

La largeur des différentes couches du mur cérébral des embryons de souris agés de 16 jours a été mésurée apres l'exposition des mères souris à une température hyperthermique de 43°C pour 10 minutes dans un fourneau de laboratoire, pendant la période de la ferméture du tuyau neural. Les mères souris ont été exposées dans le 7eme jour et demi ou le 8eme jour et demi après l'appariation. Un groupe de controle de souris a été mis dans le fourneau pour 10 minutes à une température ambiante (28°C).

A 43°C de la temperature du fourneau, la température du noyau des mères souris a été augmentée de 2-3°C ($P \leq 001$). Pour les souris qui ne sont pas enceintes, ce dégré de la chaleur résulte en une hyperthemie sontenue aux environs de six minutes et un retour a une température normale 15 minutes apres etre retirées du fourneau. Il n'y avait pas des malformations congénitales du système nerveux central. La faille moyenne des mise-bas, le pourcentage des resorptions et les poids embryonnaires fixes ont été similaires dans le groupe experimental et dans le groupe de controle.

La largeur de la plague corticale était significativement reduite dans tous les endroits mésurés ($P \leq 0.001$). La largeur de la couche matrice a été reduite seulement dans la partie fronto-parietale.

Les résultats suggerent que cette dose sub-tératogénique de la tension hyperthermique réduit la population des neurones dans le cortex cérébral.

Il se peut que cela soit le substrat structural pour le délabrement du fonctionnement chez les animaux auxquels la chaleur est appliqueé au cours du développement prénatal.

Correspondence: M. Tayo Shokunbi, Department of Anatomy, University of Ibadan, Ibadan, Nigeria.

Introduction

The evidence from retrospective studies in humans suggests that the development of the central nervous system may be adversely affected by maternal hyperthermia in pregnancy. Associations have been established between pyrexia in pregnancy and the occurrence of such central nervous system (CNS) malformations as spina bifida[1,2] and cranium bifidum[3-5]. Definitive proof is lacking largely because it has not been possible to dissociate the teratogenic effects of pyrexia from those of the diseases of which pyrexia constitutes a symptom and from maternal metabolic changes resulting from hyperthermia. However, in laboratory mammals, the embryopathic consequences of heat stress applied during the critical phase of CNS morphogenesis have been investigated.

Several techniques have been utilized to heat-stress the embryo. These include exteriorization

and exposure of the uterine horn in the rat[6], partial immersion of the pregnant mice in heated water[5], dry heating in a thermostatically controlled oven[7], and *in vitro* rat embryo culture at controlled but elevated temperature[8]. Cranial neural tube defects such as microcephaly, anencephaly and exencephaly are commonly produced. In affected rat embryos, the telencephalic mantle characteristically contains areas of cell necrosis[6]. In the guinea pig and the mouse, neuroepithelial cells in mitosis are particularly prone to necrosis within the first 24 hours of heat stress and manifest a transient interruption of the cell-cycle before prophase. This is followed by acceleration of mitotic activity and apparent recovery[5,9]. Unaffected litter mates have been shown to perform poorly at learning tasks, suggesting that sub-teratogenic heat stress may have a lasting morphologic effect on the brain.

In our study, we have examined the cortical plate thickness in the frontal, parietal and occipital cortices of embryos subjected to brief hyperthermia in utero, but not manifesting any gross malformations of the cerebrum.

In this way, we sought to determine whether non-teratogenic heat stress exerts a lasting effect on the proliferative capacity of the developing brain, as assessed by measurement of the thickness of the different layers of the cerebral mantle.

Materials and methods

Female Swiss albino mice (age 8-10 weeks) of an unspecified strain randomly bred and maintained in a closed colony in the institution's animal house facility were used for this study. Prior to mating, they were acclimatized for two weeks in the laboratory holding room in an ambient temperature of 28°C and with a light-dark cycle of approximately 13:11 hours. Tap water and laboratory mouse chow were provided *ad libitum*. The animals were housed five per cage. For mating, males were introduced overnight and females were checked for vaginal plug at 9 am next morning. Coitum was timed from the midnight preceding the morning a plug was found. Pregnant dams were randomly allocated to one of three groups: Exposure to 43°C in a dry oven for 10 minutes at

(a) day 7.5 post coitum (pc);
(b) day 8.5 p.c; and
(c) exposure to 28°C for 10 minutes in the same oven at day 8 p.c.

The last group served as the control group.

The core temperature was measured transvaginally before and after heat exposure. The animal was gently restrained and the bulb of a mercury thermometer was inserted completely into the vagina. The temperature was recorded after it had remained stable.

The animals were sacrificed at day 16 p.c. by cervical dislocation. The numbers of implants and resorptions were recorded. Under a dissecting stereomicroscope, the embryos were removed from the uterine horns and their membranes and examined for gross malformations. They were fixed in Bouin's solution for at least 24 hours. From each group, some embryonic brains were removed from the cranium and stored in 70% alcohol for 24 hours and weighed. Also from each group, 4 brains were washed for 10 minutes in physiologic saline, dehydrated in cello-solve, cleared in benzene and blocked in paraffin wax. Serial coronal sections were cut at 8μ thickness. Ten out of every 50 sections of the cerebrum were mounted on albuminized glass slides and stained in haematoxylin and eosin.

On a Leitz optical microscope, the width of the following layers (Figure 1) were measured, using an eye piece graticule previously calibrated by means of a stage micrometer: (a) the cerebrocortical mantle; (b) the matrix layer; (c) the cortical plate. For each brain, two measurements were performed on each of five sections obtained from the frontal, parietal and occipital regions. For each region therefore, a total of 40 measurements, from which mean values were calculated were obtained. The results were analysed using chi-square and student's t-tests.

Results

Heat exposure and body temperature

The core temperature in pregnant mice was elevated by 2-3°C as a result of exposure for 10 minutes to an environmental temperature of 43°C in a laboratory oven, (Table 1). Temperature measurement in non-pregnant mice revealed that this degree of heat exposure results in hyperthermia sustained for about six minutes with a gradual return towards the pre-exposure level over a period of 15 minutes (Fig. 2). The mean post exposure temperature in the experimental groups was significantly greater than that of control mice placed in the same oven, but at an ambient temperature of 28°C ($P < 0.01$).

Heat exposure and gestational outcome (Table 2)
The severity of hyperthermia utilized in this study did not result in congenital malformations of the central nervous system. The mean litter sizes and the percentage resorptions were not significantly different between the groups. This suggests that this degree of hyperthermia was not embryolethal. The mean embryonic brain weights were also similar.

Histological assessment and cerebrocortical thickness
At this stage of gestational development, three layers can be recognized in the cerebral cortex of the mouse (Fig. 1). The outermost layer consists of tightly packed cells whose longitudinal axes are oriented perpendicular to the pial surface, from which they layer is separated by an acellular thin zone. In a few areas, a few small round cells can be identified aggregated in a narrow inner stratum in this layer. Mostly, at this stage, the population is somewhat uniform. This is the cortical plate. An intermediate layer exists with a sparse cellular content containing neurons at variable distance from the outermost layer. This intermediate layer is the migratory zone through which neurons reach the cortical plate. The innermost layer, the matrix layer is the successor of the germinal neuroepithelium and lines the ventricular cavity. Daughter cells formed from mitotic division in this layer, migrate through the intermediate layer to populate the cortical plate. Therefore, the size of the cortical plate is an expression of the proliferative activity of the matrix layer.

The cerebral cortical plate, a layer populated with neurons from the matrix layer was reduced in thickness in all regions examined in the 16 day embryonic brain by *in utero* hyperthermic stress, applied shortly before and during neurulation.

Table 1 Body temperatures (°C) pre-and post exposure to heat for 10 minutes in a laboratory oven

Exposure temperature and gestational age	Body temperature (mean + SD)*	
	pre-exposure	post-exposure
28°C 8 days post coitum	36.7 ± 0.6	36.8 ± 0.6
43°C $7\frac{1}{2}$ days post coitum	36.9 ± 0.3	39.8 ± 0.4**
43°C, $8\frac{1}{2}$ days post coitum	37.1 ± 0.2	39.3 ± 0.2**

* Each value is the mean from four mice
** Significantly elevated. P <0.001

Table 2 Gestational outcome at different exposure schedules

	28°C, day 8 post coitum (control)	43°C day $7\frac{1}{2}$ post coitum	43°C day $8\frac{1}{2}$ Post coitum	
No. of litters	5	8	7	
Mean litter Size + SD	6 ± 2.3	8 ± 1.7	7 + 3.0	
Total no.of implants		26	72	54
No. of resorptions (%)	1 (14)	6 (8)*	4 (7)*	
CNS malformation (%)	0 (0)	0 (0)	0 (0)	
Embryonic Brain Weight (mg) (mean ± SD)	52.1 ± 3.5 n = 10	48 ± 3.0** n = 23	47 ± 4.3*** n = 19	

* P > 0.3
** P > 0.1
*** P > 0.5
Compared to controls

Normally, the entire cerebral mantle is wider in the frontal than it is in the parieto-occipital regions. Although, evident on ordinary visual assessment, this antero-posterior gradient was confirmed by actual measurements. The same trend was true of the cerebral cortical plate and matrix layer (Tables 3, 4, 5). Furthermore, the gradient was not altered by in utero heat stress. In regions of the brain examined, the cerebral cortical plate was smaller in heat-stressed embryos, than in controls (Table 3). There was no difference in the degree of reduction of this layer between embryos heat-stressed pre-neurulation and those heat stressed during neurulation. The width of the matrix layer was reduced in heat-stressed embryos only in the frontal and parietal regions. With respect to the total mantle thickness, a reduction was apparent only in the parietal region.

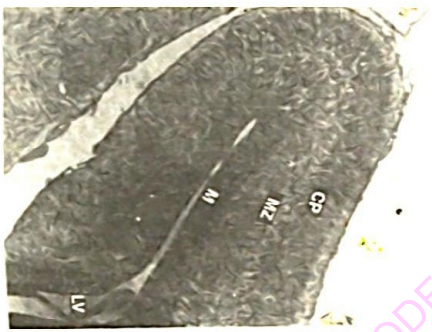

Fig. 1A coronal section of the frontal cerebral mantle of a 16-day mouse embryo. There are three major zones: the matrix layer (M) adjacent to the lateral ventricle (LV); an intermediate migratory zone (MZ); and a superficial cortical plate (CP). Haematoxylin and eosin x 200

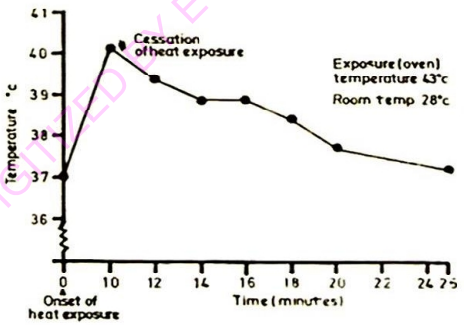

Fig. 2An illustration of the pattern of the core temperature of non-pregnant female mice exposed to an ambient temperature of 43°C for 10 minutes in a dry oven. Each dot is the mean of measurements from two mice.

Discussion

The teratogenic effect of hyperthermia on the developing nervous system appears to be specific for the cranial end of the neural tube. Despite the differences in methods of heating the embryo in laboratory animals[5-8], the most frequently encountered malformations are excencephaly, anencephaly and microcephaly. A variation in susceptibility to hyperthermia has been described, even within the same species, and it has been suggested that this may be genetically determined[10]. Because functional impairment may occur at maturity in apparently histologically normal animals[11], there is the likelihood that quantitative changes in the cell population of the cerebral cortex might exist.

The results of our study indicate that this is indeed the case: hyperthermia occuring during neurulation reduces the cell population of the cerebral cortical mantle in the pre-natal period. The development of growth, learning and behaviour has been shown to be retarded in mice[12], guinea pigs[13], and humans[14] by repetitive hyperthermia applied in the post-neurulation period. Our data raise the possibility that a similar disability can be produced when the embryo is heat-stressed during neural tube closure. It will be of considerable interest to investigate post-natal behaviour and learning in such embryos. Nevertheless, the implication of our results to human brain development is clear: brief unrecognised exposure of the embryo to pyrexia during neural tube closure may interfere with the formation of the full population of cells in the cerebrum. Whether post-natal compensation can occur is uncertain. However, the possibility that such an insult may cause intellectual impairment is not remote.

Within the neuroepithelium, cells in motosis are particularly vulnerable to the effect of heat[5,9]. The cardinal histologic features observed include death of cells in metaphase, preceded by clumping, fragmentation and extrusion of nuclear material. Examination of proportions of cells in different phases of the cell cycle revealed that a few pre-metaphase cells are also affected, and that mitosis is retarded. In single exposure experiments, these changes were rapidly succeeded by clearance of necrotic debris and re-appearance of normal mitotic figures twelve hours after cessation of heat stress. Our findings imply that the recovery is not total, and that the proliferative potential of the neuroepithelium is in some way permanently altered.

Table 3 Embryonic cortical plate thickness (μ) following exposure of pregnant mice to 43°C for 10 minutes

	Gestational age (days post coitum) at exposure		
Brain Region*	Control	day $7\frac{1}{2}$	day $8\frac{1}{2}$
Frontal	182.6 ± 13.8	129.3 ± 23	139.7 ± 24.2
Parietal	144.7 ± 12.1	86.9 ± 10.5	98.5 ± 13.8
Occipital	104.5 ± 9.4	74.3 ± 12.7	83.1 ± 4.4

* For each region the value in the control group is significantly greater than those in the experimental groups, $P < 0.001$

Table 4 The thickness (μ) of the matrix layer in 16-day old embryos exposed to hyperthermia for 10 minutes *in utero*

Brain Region	Temperature and gestational age (days post coitum)		
	28°C, day 8 (control)	43°C, day $7\frac{1}{2}$	43°C, day $8\frac{1}{2}$
Frontal	112.8 ± 13.2	124.3 ± 33^{bb}	138.6 ± 27.5b
Parietal	80.9 ± 7.2	57.7 ± 7.2^{b}	55.0 ± 0^{b}
Occipital	55.0 ± 0	52.8 ± 7.3^{a}	55.0 ± 0^{a}

a. Not significantly different from control values
b. Significantly smaller than control values, $P \leq 0.001$
bb. Weakly significantly smaller than control values $P \leq 0.08$.

Table 5 Effect of *in utero* hyperthermia on cerebral mantle thickness (μ) in 16-day old embryos

Brain Region	Temperature and gestational age at exposure		
	28°C, day 8 (control)	43°C, day $7\frac{1}{2}$	43°C, day $8\frac{1}{2}$
Frontal	639.7 ± 113.3	620.4 ± 71.0^{a}	676.5 ± 99.0^{a}
Parietal	481.3 ± 29.7	345.4 ± 25.9^{b}	379.5 ± 32.5^{b}
Occipital	286.0 ± 22.6	276.6 ± 7.2^{a}	288.8 ± 13.8^{a}

a. Not significantly different from control values
b. Significantly smaller than control values, $P < 0.001$

This is supported by studies of cultured lung tissue from Chinese hamsters which revealed that a proportion of the pool of proliferating cells is lost as a result of thermal injury[15]. It can be argued that the cells with intact nuclear envelope (i.e. not dividing) within the guinea pig neuroepithelium that were damaged by heat[9] were lost and that such losses can reduce the size of the dividing pool.

Several mechanisms have been proposed to explain the effect of heat on rapidly dividing cells such as those of the neuroepithelium. In *Tetrahymena*, the turnover of RNA associated with mitosis is reduced by heat treatment[16]. In sea urchins, Zeuthen[17] showed that heating fertilized eggs to 27°C prevented splitting of chromatids in cells that have already lost their nuclear envelopes, and resulted in reversal of the cell to a single nucleus (i.e. interphase) status. The nature of this change suggests that it may have been caused by damage to the spindle microtubules. The fact that the thermodynamic characteristics of

protein denaturation correlate well with thermal cell death rates[18] strongly supports protein denaturation as a major mechanism of hyperthermic cell injury. Finally, in mice, the reduction in size of the cortical plate induced by maternal protein-calorie malnutrition was shown to be associated with a reduced proliferative activity in the matrix layer, as evidenced by prolongation of the time for DNA synthesis and cell generation. Based on the foregoing, a plausible sequence of events in our animals would be as follows: thermal injury to the neuroepithelium during neurulation results in some cell loss and slowing of cell generation from the surviving proliferative pool. Consequently, proliferative activity is reduced in the neuroepithelium and its successor the matrix layer. This is expressed as a reduction in the size of the cortical plate, a layer formed by cell division in and migration from the matrix layer.

In the matrix layer, proliferative activity is of greater relevance than size and there is really no direct or proportional relationship between the two[19,20]. Moreover, the matrix layer recedes shortly after birth. Therefore, not much significance can be attached to the observation in our study that this layer is not uniformly reduced by hyperthermia. This may indeed simply reflect the variation in rates of regression of this layer.

Brain weight deficits were not observed in this study. It should be noted that reports of such deficit have emanated from experiments in which the heat insult was repeatedly applied[12,21,22], including periods beyond the time of neural tube closure. These studies and ours indicate that the reduction in brain weight required a summation of effect, but that in its absence the cerebral cortical plate in the late pre-natal mouse is not completely normal.

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References

1. Chance P F, Smith D W. Hyperthermia and meningomyelocele and anencephaly. Lancet 1978; i: 769-770.
2. Layde PM, Edmonds LD, Erickson JD. Maternal fever and neural tube defects. Teratology. 1980; 21: 105-108.
3. Miller P, Smith DW, Shepard TH. Maternal hyperthermia and a possible cause of anencephaly. Lancet 1978; i: 519-521.
4. Fisher NL, Smith DW. Occipital encephalocoele and early gestational hyperthermia. Pediatrics 1981; 68: 480-483.
5. Shiota K. Induction of neural tube defects and skeletal malformations in mice following brief hyperthermia *in utero*. Biol. Neonate 1988; 53: 86-97.
6. Skreb B, Frank Z. Developmental abnormalities in the rat induced by heat shock. J. Embryol exp morp. 1983; 11: 445-457.
7. Seller MJ, Perkins-Cole KJ. Hyperthermia and Neural tube defects of the curly-tail mouse. Journal of Craniofacial Genetics and Developmental Biology 1987; 7: 321-330.
8. Cockroft DL, New DAT. Effects of hyperthermia on rat embryos in culture. Nature. Lond. 1975; 258: 604-606.
9. Edwards MJ, Mulley R, Ring S, Wanner RA. Mitotic cell death and delay of mitotic activity in guinea pig embryos following brief maternal hyperthermia. J. Embryol. exp. morphol. 1974; 32: 593-602.
10. Finnel RH, Moon SP, Abbott LC, Golden JA, Chernoff GF. Strain differences in heat-induced neural tube defects in mice. Teratology 1986; 33: 247-252.
11. Lyle JG, Edwards MJ, Johson KM. Critical periods and the effects of pre-natal heat stress on the learning and brain growth of mature guinea pigs. Biobehav. Rev. 1977; 1: 1-13.
12. Shiota K, Kayamura. Effects of pre-natal heat stress on post-natal growth, behaviour and learning capacity in mice. Biol. Neonate 1989; 56: 6-14.
13. Edwards MJ, Lyle JG, Johson KM, Panney RHC. Prenatal retardation of brain growth by hyperthermia and the learning capacity of guinea pigs. Dev. Psychobiol. 1974; 7: 579-584.
14. Pleet H, Graham JM Jr, Smith DW. Central nervous system and facial defects associated with maternal hyperthermia at 4 to 14 weeks gestation. Pediatrics. 1981; 67: 785-789.
15. Johnson HA, Pavelec M. Thermal injury due to normal body temperature. Am. J. Path. 1972; 66: 557-564.
16. Moner JG. Temperature, RNA synthesis and cell division. Exp. Cell. Res. 1967; 45: 618-630.
17. Zeuthen E. Inhibition of chromosome separation in cleaving *Psammechinus* eggs by elevated temperature. Exp. Cell. Res. 1972; 72: 337-344.
18. Rosenberg B, Kemeny G, Switzer RG, Hamilton TC. Qualitative evidence for protein denaturation as the cause of thermal death. Nature. Lond. 1971; 232: 471-473.
19. Shimada M, Yamano T, Nakamura T, Morikawa Y, Kusinoki T. Effect of maternal malnutrition on matrix cell proliferation in the cerebrum of mouse embryo: An autoradiographic study. Pediat. Res. 1977; 11: 728-731.

20. Hoshino K, Matsuzawa T, Murakami U. Characteristics of the cell cycle of matrix cells in the mouse embryo during histiogenesis of the telencephalon. Exp. Cell. Res. 1973; 77: 89.
21. Edwards MJ. Congenital defects in guinea pigs following induced hyperthermia during gestation. Arch. Path. 1967; 84: 42-48.
22. Edwards MJ. Penny RHC, Zevnik I. A brain cell deficit in newborn guinea pigs following pre-natal hyperthermia. Brain Research 1971; 28: 341-345.

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