

# Hyperthermia, teratogenesis and the heat shock response in mammalian embryos in culture

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**ABSTRACT** Hyperthermia is a recognized teratogen in animals and there is strong evidence that it also causes significant damage to human embryos. Studies with induced hyperthermia in pregnant animals defined the defects which are produced, the susceptible stages of development, and threshold doses of heat required to cause defects. The *in vivo* experiments lacked precision because of variability of embryonic development at a given conceptual age, varying maternal responses to agents causing temperature elevations, the difficulty in measuring embryonic temperature and the possibility that defects were caused by toxic changes in maternal metabolism. These variables were eliminated by the use of postimplantation whole rat and mouse embryo cultures, which were exposed to various doses of heat at closely defined stages of development. The studies showed that heat acts directly on embryos and that elevations of 2°C and greater sustained over early rat organogenesis cause defects mainly by causing apoptotic cell death especially in the developing central nervous system. A moderate, non damaging exposure is followed within 15 min by protection for up to 8 h against a more severe and otherwise teratogenic exposure. The protective heat shock response is accompanied by a reduction of normal protein synthesis and concurrent synthesis of heat shock proteins (HSP90, 71, 47, 27). Most HSP in these families are also present constitutively in embryos, probably having important roles in protecting newly synthesized proteins from aggregation and facilitating folding into their normal functional configurations. The appearance of induced HSP and hsp mRNA at known sites of thermal damage suggests a protective role. Heat induced cell death by apoptosis is a feature of teratogenic damage to the developing brain. Apoptosis could be a by-product of a damaging heat exposure because of a priority favoring induction of the heat shock response over the normal gene program for organogenesis, survival being achieved at the expense of normal development.

**KEY WORDS:** *heat stress, embryo culture, heat shock proteins, developmental defects, thermotolerance*

## Introduction

### *Incidence and causes of birth defects*

Records from many sources indicate that approximately 3% of newborn children have a developmental defect requiring medical attention, and approximately one-third of these conditions can be regarded as life threatening. With increasing age, the number of defects detected is more than doubled (Shepard, 1989). The emotional and financial cost of birth defects is enormous; approximately one-half the children in hospitals are there because of a birth defect. In most instances the cause of the defect remains unknown, about 25% are genetic in origin and less than 10% can be ascribed to a teratogenic agent. Although Shepard (1989) lists only 36 known and 13 possible human teratogens, there are at least 1200 agents known to cause defects in animals. This discrepancy needs to be kept in mind when reviewing the results of experimental animal research. A small minority of known animal teratogens are also known to cause birth defects in humans. The

great majority of the non-infectious human teratogens, under the correct conditions of dose and timing of dose, have been shown to cause birth defects in one or more species of animals. Human teratogens have usually been detected by clinical observation or less frequently by epidemiological surveys of children with defects and subsequently, the mechanisms and conditions of teratogenicity have been studied in animals.

### *Hyperthermia as a teratogen*

Hyperthermia is an exception to this generalization. Animal research showed that it was teratogenic in many species, including primates, and the conditions and mechanisms of the action of hyperthermia were defined in animals before a systematic study of its effects was made in humans. Subsequent human clinical observations and epidemiological studies indicated that hyperthermia is also a human teratogen although general acceptance that a naturally occurring environmental agent such as heat could be teratogenic has been slow. The volume of clinical, epidemiological

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and experimental evidence on heat as a teratogen is now very large (review by Edwards *et al.*, 1995). Hyperthermia refers to a higher than normal body temperature. It can be caused by many conditions and agents including febrile infections, heavy exercise, a hot and humid environment, exposure to saunas, hot tubs, electromagnetic radiations, microwaves and ultrasound and many drugs (such as phenothiazines, amphetamines, tricyclic antidepressants, antihypertensive drugs, cocaine, LSD), and organic compounds (such as organophosphates, dinitrophenols) particularly when taken in a hot environment (Lomax, 1987). These agents and conditions overcome or alter the physiological homeostatic mechanisms which hold body temperatures at relatively stable levels. Most deep core body temperatures of mammals fall between 37°C and 40°C and remain relatively stable under widely variable environmental conditions of cold and heat. Normally, the deep (core) body temperature is lowest during periods of inactivity and sleep, increasing on arousal, physical activity and feeding, the usual range being 1°C on either side of the average for the species.

The relatively high and stable body temperatures of mammals confer many advantages in survival compared with poikilothermic animals. It could be inferred that evolutionary pressures should result in even higher body temperatures unless prevented by some biochemical or physiological barriers. It has been suggested that the deleterious effects of heat on spermatogenesis (Cowles, 1965) and cellular proliferation in embryos (Edwards, 1979) are two such barriers. Although it has been recognized for many years, that heat during early pregnancy causes increased rates of embryonic resorption in many species of animals, the other effects of heat in causing defective development have only recently been explored in detail.

The type of defects caused by heat depends largely on the stage of embryonic development at the time of exposure and the severity depends largely on the "dose" of heat, which is a product of the temperature elevation and the duration of elevation (see below). Exposure of pre-implantation embryos is commonly followed by embryonic death and resorption in a wide range of species (Bell, 1987) and these deaths can be caused by quite modest temperature elevations. More severe exposures of post-implantation embryos or fetuses are required to cause embryonic or fetal death and resorption or abortion. After implantation, severe exposures are followed by prenatal death and resorption or abortion while defective embryonic development follows less severe exposure. Experimental production of defects by heat, with low levels of resorptions and abortion, can be achieved by maintaining rigid control of the environmental temperature and the duration of exposure with frequent monitoring of the maternal temperature. During non-experimental exposures such as in infectious fevers, saunas, hot tubs and drugs, there is not the same level of control of the dose of heat and it has been suggested that abortion could be the most common adverse outcome in women (Graham and Edwards, 1988).

The "dose" of heat is a product of the elevation of temperature and the duration of elevation. There is no simple measure for dose, but a number of authors have approached the problem of threshold dose by finding the shortest exposure time at a given elevation of temperature for the production of a defect (Lary *et al.*, 1983; Germain *et al.*, 1985; Walsh *et al.*, 1987; G.L. Kimmel *et al.*, 1993). These studies show that as the exposure temperature is increased, the time required to cause a defect is reduced logarithmically.

## Developmental defects caused by hyperthermia

### Experimentally induced defects

Defects have been produced experimentally by hyperthermia caused by a variety of methods. In chickens it was caused by elevated temperatures of incubation (Dareste, 1877; Alsop, 1919; Nilsen, 1985). In rats, exposure in hot air incubators (Hsu, 1948; Edwards, 1968; C.A. Kimmel *et al.*, 1993), exteriorized pregnant uterine horn immersed in hot water (Skreb and Frank, 1963), diathermy (Hofmann and Dietzel, 1966), abdomen of pregnant rat immersed in warm water (Germain *et al.*, 1985; Webster *et al.*, 1985), heated embryo cultures (Cockroft and New, 1975, 1978; Mirkes, 1985; Walsh *et al.*, 1985, 1987; C.A. Kimmel *et al.*, 1993), radiofrequency radiation (Lary *et al.*, 1982, 1983; Brown-Woodman *et al.*, 1988), and ultrasound exposure of cultured embryos (Angles *et al.*, 1990), have been used. In mice, hyperthermia was caused by hot air incubators (Lecyk, 1966; Hirsekorn, 1980), abdomen of pregnant mice immersed in warm water (Webster and Edwards, 1984; Finnell *et al.*, 1986; Shiota, 1988), and microwave exposure (Fukui *et al.*, 1992). In guinea pigs it was produced by exposure in hot air incubators (Edwards, 1967, 1969a,b; Edwards *et al.*, 1995). In hamsters in hot air incubators (Kilham and Ferm, 1976; Ferm and Kilham, 1977; Ferm and Ferm, 1979). In rabbits, fever was induced by injection of milk (Brinsmade and Rubsaamen, 1957), and endotoxin (Hellmann, 1977). Exposure in hot air chambers was used in sheep (Hartley *et al.*, 1974), pigs (Done *et al.*, 1982), and in monkeys (Poswillo *et al.*, 1974; Hendrickx *et al.*, 1979).

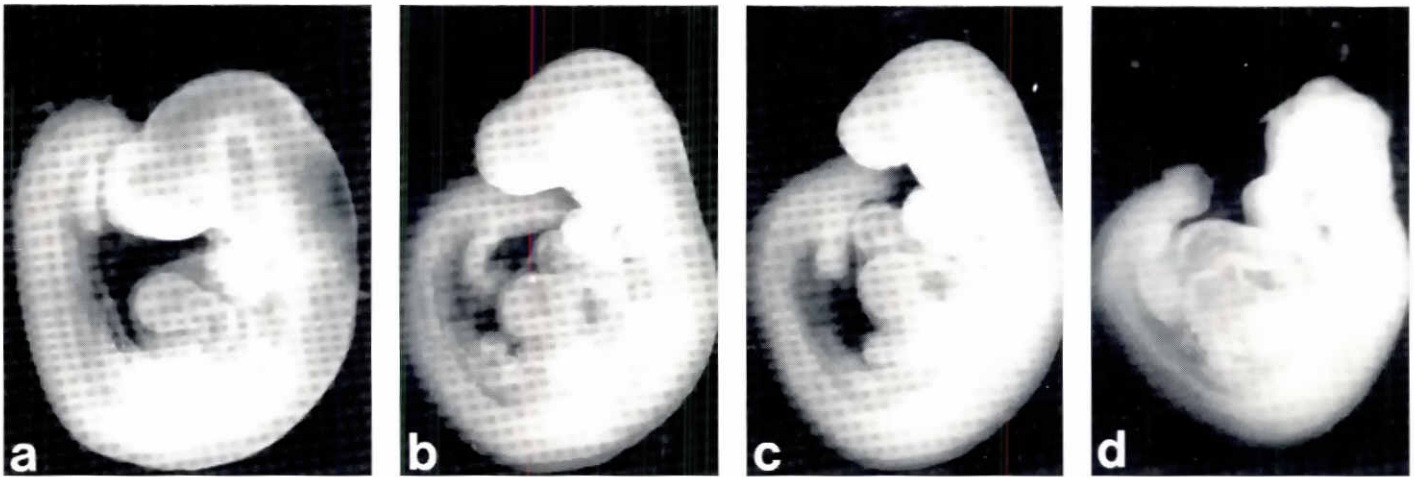
In a number of studies there was evidence of synergism between minimally teratogenic doses of heat and minimally teratogenic doses of other agents including vitamin A (Ferm and Ferm, 1979), arsenic (Ferm and Kilham, 1977), lead (Edwards and Beatson, 1984), ultrasound (Angles *et al.*, 1990), alcohol (Shiota *et al.*, 1988), endotoxins (Hilbelink *et al.*, 1986) and X rays (Nakashima *et al.*, 1991).

The defects induced experimentally at susceptible stages of development include neural tube defects, cranio-facial defects, microphthalmia, heart defects, coloboma, kyphosis, scoliosis and skeletal defects when exposure occurs about the time of neural tube closure. Cataract, talipes, hypodactyly, micrencephaly, renal and dental agenesis, exomphalos, cranial nerve defects, and behavioral abnormalities, follow exposure at later stages of embryogenesis after the closure of the neural tube and arthrogryposis multiplex congenita can follow exposure at about the end of organogenesis and rapid neuronal proliferation (Edwards, 1986).

### Human studies

Human studies have shown a number of similar defects to be associated with a maternal hyperthermic episode. Neural tube defects were prominent (Shiota, 1982; Hunter, 1984; Milunsky *et al.*, 1992), and it was estimated that approximately 10% were due to maternal hyperthermia at the time of tube closure (Graham and Edwards, 1988). Other defects were microphthalmia (Fraser and Skelton, 1978; Spraggett and Fraser, 1982), microcephaly, neuronal heterotopias, micropenis, micrognathia, mid-face hypoplasia, cleft lip/palate, external ear anomalies, mental deficiency, hypotonicity, neurogenic contractures including talipes and arthrogryposis, seizures (Pleet *et al.*, 1981), Moebius syndrome (Graham *et al.*, 1988), and Hirschsprung disease (Lipson, 1988).





**Fig. 1.** Effects of increasing duration of exposure at 42°C on development of 9.5 day rat embryos in culture. (a) Control embryo at 11.5 days, (b) 10 min at 42°C, (c) 15 min at 42°C, (d) 20 min at 42°C. Note the increasing severity of damage, particularly to the head.

#### Limitations of whole animal *in vivo* studies

The experimental *in vivo* studies provided information about the types of induced defects, their approximate stages of greatest sensitivity during embryogenesis, mechanisms of causing defects and approximate thresholds of maternal temperature elevation and duration of elevation. However, many studies lacked precision in a number of areas. The periods of susceptibility to defects can be quite brief (Webster and Edwards, 1984; Finnell *et al.*, 1986). In *in vivo* studies, the wide variability of embryonic development at a given conceptual age, even within litters, appeared to result in irregular expression of the effects of heat especially following a single brief exposure. In contrast, by using embryo culture techniques, the stage variability can be minimized by selecting embryos at a precise, identifiable stage of development, for example, the early pre-somite head fold stage at 9.5 days (Walsh *et al.*, 1987), embryos at 6-10 somites with yolk sac circulation and heart beat (Mirkes, 1987) or at 10-12 somites (Kimmel *et al.*, 1993).

Another problem with *in vivo* studies is the difficulty in estimating the dose of heat delivered through the mother to the embryo. The approximate embryonic dose can be estimated by monitoring the rectal temperature of the mother during the heating and cooling phases (Edwards, 1969a). In embryo culture studies, it is possible to apply a precise temperature to the embryo, with short heating and cooling phases and to maintain this temperature for a precise duration.

In clinical studies, it is often difficult to separate the direct and indirect effects of an agent on the embryo. In instances such as viral infection causing maternal fever, a teratogenic effect could be due to viral infection of the embryo, metabolic changes in the mother affecting the embryo or direct action of hyperthermia on the embryo. The embryo culture technique can eliminate the maternal effects.

#### Embryo culture studies of the teratogenicity of hyperthermia

##### Initial studies with 9.5 and 10.5 day rat and 8 day mouse embryos

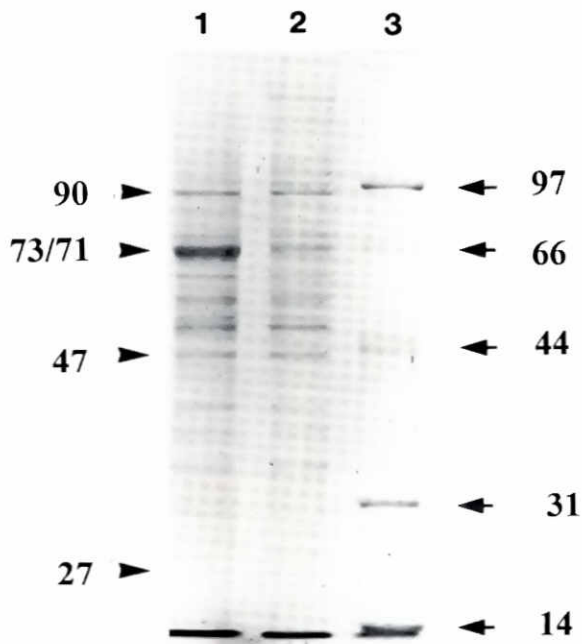
Cockroft and New (1975, 1978) were the first to use elevated temperatures on rat embryos in culture. Embryos of the CFHB

strain were explanted on day 9.5 of gestation (egg cylinder, early neural fold stage) and cultured for 2 days at 38°C (controls), 40 or 41°C. Other heated groups were exposed for 12 or 23 h of the culture period to 40, 40.5 or 41°C, and for the rest of the culture at 38°C. Culture at 40°C for 2 days resulted in overall growth similar to controls but blood circulation was disturbed in a portion and about half had small developmental abnormalities, including a number with apparent microcephaly. Two methods were used to clarify the effect of culture at 40°C on brain and head growth. In the first method, embryos grown at 38°C were paired with an embryo at 40°C on the basis of total body protein content. The amount of protein in the heads was measured separately. The mean total body protein content of each group was identical but the head:body ratio of the 40°C group was significantly less than that of the control group. The other method matched individual embryos from each group on the basis of crown-rump length and measured the length of the head and the length, height and width of the telencephalon. The length of the head and the length and height of the telencephalon were significantly less in the 40°C group compared with the 38°C group.

At 40.5°C over the 2 days, growth was retarded, somite numbers were less and over half the embryos were obviously microcephalic. Exposure to 41°C for the whole culture period caused severe problems including very poor growth and somite formation, microcephaly, neural tube defects, enlarged hearts and pericardial edema. In experiments in which embryos were cultured at 41°C for 12 hour periods between 9.5 and 11.5 days, development was most vulnerable to damage during late day 9 to early day 10.

Walsh *et al.* (1985, 1987) used 9.5 day (early head fold, pre-somite) rat embryos, which were cultured using a modification of the method of New *et al.* (1973) at 38.5°C throughout the 2 days of the culture period (controls). After 2.5 h at 38.5°C some were exposed to various regimes of hyperthermia for up to 80 min and then returned to the cabinet at 38.5°C for the remainder of the 2 day culture period. In dose-response experiments, exposure to 43 or 43.5°C for as little as 0.5 min caused significant deficits of protein accumulation measured on day 11.5. Microphthalmia, neural tube defects and reduced forebrain were caused by the lowest doses. A temperature of 43.5°C (an elevation of 5°C) for 2.5 min, an





**Fig. 2. Protein synthesis in 9.5 day rat embryos. (Lane 1)** Heat shocked embryos at 1 h following 42°C for 10 min. **(Lane 2)** Control. **(Lane 3)** Markers. Twelve embryos were labeled with [<sup>35</sup>S] methionine for 60 min before analysis, 2 µg of protein was loaded per lane, separated by polyacrylamide gel electrophoresis and analysed by fluorography and optical densitometer. Note the induction of *hsp71* and constitutive synthesis of HSP90, 47 and 27 and HSC73 indicated by arrows (data from Walsh et al., 1987, 1993; Li, unpublished).

elevation of 4.5°C for 7.5 min, 4°C for 10 min or 3.5°C for 40 min caused one or more defects, showing that as the temperature is increased, the time required to cause defects is reduced logarithmically. Also, as time at a given temperature increases, the severity of defects is increased (Fig. 1). These findings match those of Germain et al. (1985) who used similar temperature and duration combinations in rat embryos *in vivo*. Pretreatment at 42°C for 10 min significantly protected against a subsequent exposure of 43°C for 7.5 min which in unprotected embryos caused severe damage.

Mirkes (1985, 1987) studied the effects of exposures of 10.5 day rat embryos in culture to 42°C or 43°C for 30 min and 42°C for 30 min followed in 1 h by an exposure to 43°C for 30 min. The treatment at 42°C had no apparent effect on development whereas 43°C for 30 min caused severe mortality and defects in the surviving embryos. Embryos given 42°C for 30 min followed by 43°C for 30 min had some protection against mortality and defects.

Comparisons of results of *in vitro* with *in vivo* exposures from the same laboratory are few, but valuable. By exposing day 10 (10-12 somite) rat embryos *in vitro*, after 2 h at 38°C, to temperatures of 42°C or 43°C for 10-25 min, G.L. Kimmel et al. (1993) found a dose-related general inhibitory effect on growth in all systems, especially systems that were developing most rapidly at the time of the exposure. The embryos were evaluated on day 11 using a modified Brown and Fabro (1981) method. A similar exposure *in vivo* on day 10 (C.A. Kimmel et al., 1993) resulted at term, in a very high rate of similar skeletal defects of the thoracic vertebrae and ribs, but few defects of the head. The authors suggested that this disparity in

response could be due to a greater dose of heat in the *in vitro* system in which embryos were kept at 42°C for at least 10 min which was twice as long as the *in vivo* exposure. Their results showed that in the *in vivo* experiments, it took at least 50 min to achieve the required temperature. When estimating the dose of heat *in vivo*, prolonged heating-up and cooling-down phases should be considered for inclusion in the dose because a significant amount of heat can be delivered during these periods. The durations of the heating and cooling phases *in vitro* usually occupy only 2-3 min each. It also appears possible that the disparate results could be due to a high prenatal mortality of severely defective embryos *in vivo*. In addition, the authors pointed out that *in vitro* embryos might have recovered from many of the minor developmental deficits by subsequent compensatory growth *in utero*.

Angles et al. (1990) used 9.5 day rat embryos in culture to test the effects of 5, 15 or 30 min of pulsed ultrasound (SPTA intensity of 1.2 W/cm<sup>2</sup>, similar to diagnostic Doppler). Examination at 11.5 days showed that insonation for 30 min caused only a small deficit in somite number, equivalent to a 2 h delay in development. Embryos given similar insonation for 15 min in culture medium at 40°C (+1.5°C), had a significant reduction in total protein and also in the head:body surface area ratio. Insonation for 15 min at 38°C, or culture at 40°C for 15 min alone had no detectable effect on development.

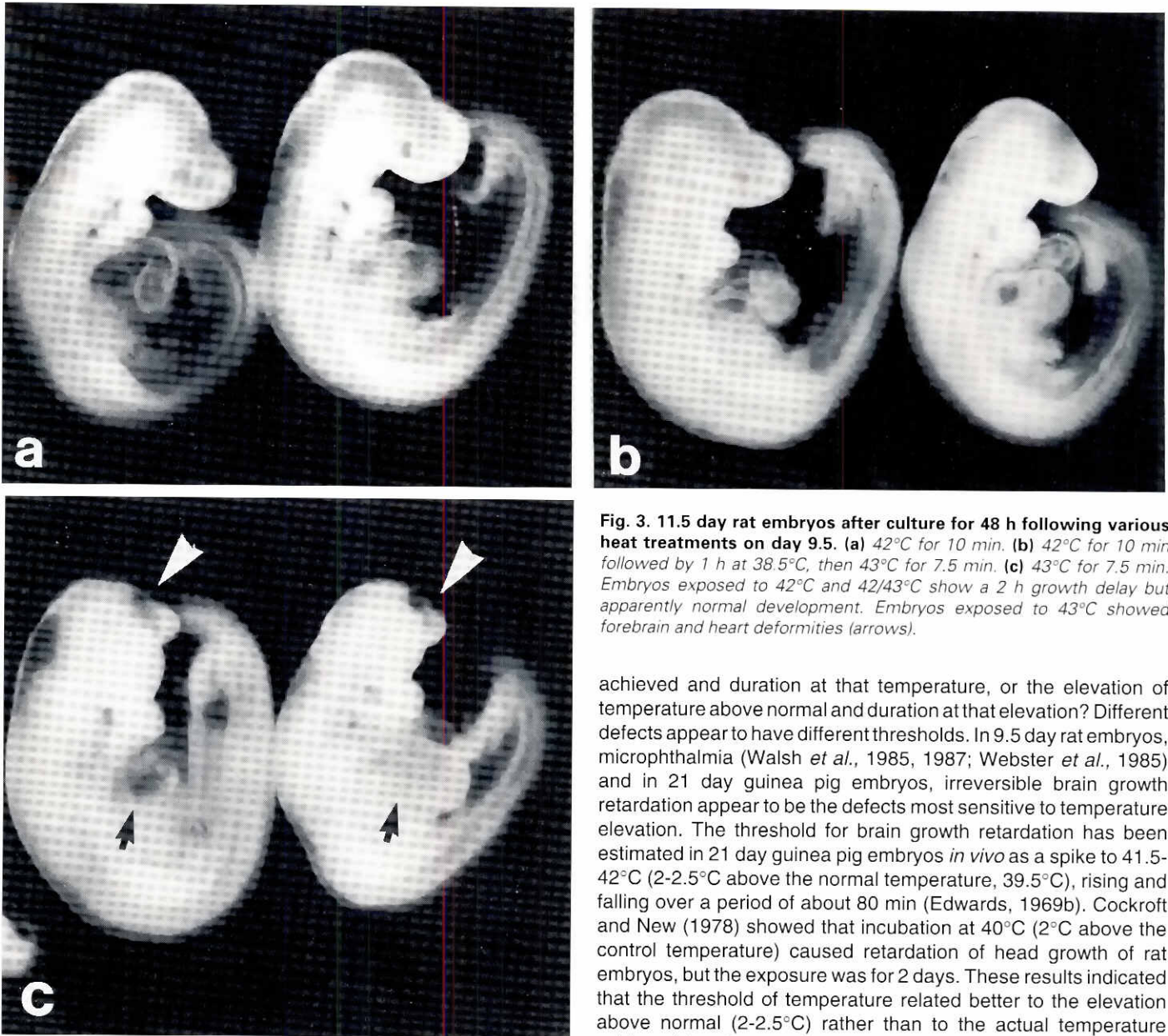
Nakashima et al. (1991) used cultured 8 day (3-5 somite) mouse embryos (C57BL/6 Cr Slc female X C3H/He Slc male strains) to compare the individual and combined effects of X-irradiation and hyperthermia on early development. Dose response studies showed that 0.3 Gy of X rays given at 1 h after the start of culture caused no detectable changes in development at the end of the 40 hour culture period. Doses between 0.6-2.0 Gy caused progressively more damage, with 10% of embryos given 0.6 Gy showing microphthalmia and 77% of embryos given 2.0 Gy showing open neural tubes and 100% microphthalmia. Exposure to 43°C for 5 min caused minor retardation of protein accumulation and somite formation. Embryos exposed for 10 min had 28% open neural tube and 78% microphthalmia, increasing to 100% open neural tube, 100% anophthalmia and 100% heart defects when given a 20 min exposure. Control embryos were incubated at 38°C. In terms of defective development, the 10 min exposure to 43°C corresponded roughly to 1.2 Gy of X rays. A combination of 0.3 Gy and 5 min at 43°C, which were not teratogenic when given individually, caused significant microcephaly, 17% microphthalmia and 11% heart defects.

The interactions which were found between subteratogenic doses of hyperthermia and ultrasound or X rays in cultured embryos and between hyperthermia and alcohol, endotoxins, arsenic, lead or vitamin A *in vivo* are of considerable significance. It is apparent that synergistic interactions with small and usually harmless doses of a number of agents can increase the susceptibility to damage by heat.

#### Relevance of the initial studies

The precision of the embryo culture experiments clarified a number of points which had not been possible using pregnant animals. They showed that hyperthermia acted directly on the embryo and that defects were not mediated through toxic maternal changes (Khera, 1985), although the maternal reaction might modify the embryonic response. The studies also confirmed the





**Fig. 3.** 11.5 day rat embryos after culture for 48 h following various heat treatments on day 9.5. (a) 42°C for 10 min. (b) 42°C for 10 min followed by 1 h at 38.5°C, then 43°C for 7.5 min. (c) 43°C for 7.5 min. Embryos exposed to 42°C and 42/43°C show a 2 h growth delay but apparently normal development. Embryos exposed to 43°C showed forebrain and heart deformities (arrows).

achieved and duration at that temperature, or the elevation of temperature above normal and duration at that elevation? Different defects appear to have different thresholds. In 9.5 day rat embryos, microphthalmia (Walsh *et al.*, 1985, 1987; Webster *et al.*, 1985) and in 21 day guinea pig embryos, irreversible brain growth retardation appear to be the defects most sensitive to temperature elevation. The threshold for brain growth retardation has been estimated in 21 day guinea pig embryos *in vivo* as a spike to 41.5–42°C (2–2.5°C above the normal temperature, 39.5°C), rising and falling over a period of about 80 min (Edwards, 1969b). Cockroft and New (1978) showed that incubation at 40°C (2°C above the control temperature) caused retardation of head growth of rat embryos, but the exposure was for 2 days. These results indicated that the threshold of temperature related better to the elevation above normal (2–2.5°C) rather than to the actual temperature achieved (40°C in rats and 41.5°C in guinea pigs). Except when interacting with other agents, any duration of exposure to temperature elevation of less than 2°C does not appear to cause defects in any species. At higher elevations, the duration becomes very important. Germain *et al.* (1985) found thresholds for damage to 9.5 day rat embryos *in vivo* of 41°C (+2.5°C over controls) for 60 min, 42°C (+3.5°C) for 10 min and 43°C (+4.5°C) for less than 2 min.

### The heat shock response in cultured mammalian embryos

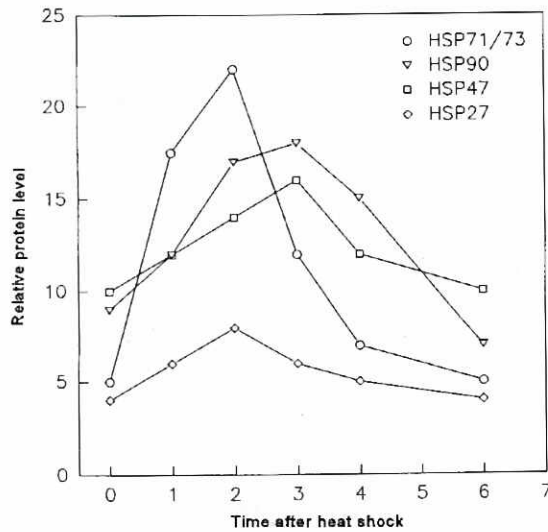
#### *The heat shock genes (hsp) and heat shock proteins (HSP)*

Elevated temperatures provoke a response which is similar in plants and animals whether multicellular or unicellular, embryonic or mature and (in animals) vertebrate or invertebrate (Lindquist, 1986). This indicates the fundamental importance of the adaptive response, known generally as the heat shock response. Ritossa

extreme sensitivity to retardation of brain growth. It is uncertain whether the deficit in head size and forebrain of the 11.5 day rat embryos would be permanent. However, the deficit in brain size found in 30 day guinea pig embryos after heating on day 21 persisted to adult life (Edwards *et al.*, 1976; Edwards, 1981). Also, retardation of growth of the head resulted from a 2°C elevation of temperature, which is well within the range occurring in fevers and other environmentally induced elevations. Embryos cultured at 40.5°C (+2.5°C) showed more severe microcephaly and retardation of development.

Because of the different experimental conditions employed in various rat embryo culture and *in vivo* studies, it is difficult to draw conclusions on a number of factors. One factor has been the definition of the threshold dose causing defective embryonic development. Is the threshold best described by the actual temperature





**Fig. 4.** HSP90, 71/73, 47 and 27 synthesis in cultured rat embryos after heat shock of 43°C for 7.5 min on day 9.5, expressed as changes relative to control values. Each time point represents data from 12 embryos which at 10-15 min after heating were labeled with  $^{35}$ S methionine for 1 h and sampled 1-6 h after the exposure. Incorporation of radioactive protein was measured by laser densitometry (data on HSP90, 71/73, 27 Walsh et al., 1989, 1993 and HSP47 Li, unpublished).

(1962) first described the onset of this reaction in heated *Drosophila* pupae in which gene activity was noted in the form of puffing patterns in their salivary gland polytene chromosomes. The subsequent research which led to the identification of the protective response used heat as the stress, hence the terms, the heat shock response and heat shock proteins. As the response can also be elicited by certain other stressful agents including hypoxia, deprivation of glucose, heavy metals, ethanol, metabolic poisons, protein denaturants, and ultrasound, it is also termed the stress response. It is characterized generally by the rapid inhibition of normal protein synthesis and the concurrent rapid, coordinated synthesis of a group known collectively as heat shock proteins (HSP) or stress proteins. The response is brought about by the activation of the heat shock genes (*hsp*), when the organism is exposed to a sufficient dose of heat or certain other stresses. After the induction of the response, the organism is more resistant to otherwise lethal or seriously damaging exposures to that, and to some other stressors.

Two major groups of stress proteins have been identified, based on the response to different stresses, the HSP and the glucose regulated proteins (GRP). The HSP are induced by hyperthermia and also by exposure to heavy metals, alcohol, metabolic inhibitors and protein denaturants. The GRP are synthesized in response to deprivation of glucose, oxygen and substances which disturb protein transport and calcium metabolism. The HSP will be covered in this review. Within the HSP, some distinct families are recognized, based on their molecular weights (kDa). The categorization of HSP by size also relates generally to their functions. The HSP families which have been studied in embryonic development are HSP90 (a family with a molecular weight about 90 kDa), HSP70, HSP47, HSP20 and ubiquitin which is the smallest HSP. Most HSP have two or more genes, an inducible copy which is induced by heat or other

stresses and a constitutively expressed cognate (HSC) which is involved in normal cellular activities.

Many stress proteins are also molecular chaperones, which facilitate polypeptide transport and the folding of newly synthesized proteins into their functional configurations but without becoming incorporated into the final product. Chaperone proteins protect newly synthesized proteins against inappropriate folding and against interactions with uncovered active surfaces on other proteins to form functionless aggregates. Newly induced HSP bind to thermally sensitive proteins in the cytoplasm while others translocate to the nucleus binding to, and protecting nuclear protein complexes (Lindquist, 1986; Schlesinger, 1990; Hightower, 1991). The HSP with chaperone functions are also known as chaperonins and include HSP90, HSP70, HSP47 and the small HSP families. It appears that heat inducible HSP and some HSC also protect and reconstitute heat damaged proteins by binding to uncovered, active sites of partially unfolded proteins, protecting them against binding randomly to similar sites on other damaged proteins. Reconstitution is possible by the orderly disengagement of the HSP and HSC to allow the assumption of the correct functional structure of the rescued protein.

The HSP90 family is present constitutively in relatively large amounts in the cytosol of normal unstressed cells. Its function is uncertain but it might act as a molecular chaperone. After heat stress, it is only moderately induced. It has transient interactions with steroid receptors, actin and tubulin and is associated with microtubules of interphase cells and the spindle of mitotic cells. GRP94, a glucose regulated protein, is a member of this family.

The HSP70 family has several members with different functions. The constitutive HSC73 (a heat shock cognate) is normally present in the cytosol and nucleus and is moderately induced by heat. Under normal conditions HSP71 is usually undetectable, or present in very small quantities. It is strongly induced by heat, being found in the cytosol and nucleus, binding to damaged proteins.

HSP47 has been identified recently as a stress protein (Nagata et al., 1988). It is a member of the serpin group, situated in the endoplasmic reticulum and binds to collagen. It is present constitutively in cells and is strongly induced by heat.

The low molecular weight family (HSP20) is represented in mammals mainly by HSP27. It is present constitutively and is strongly induced by heat, and is a molecular chaperone, particularly of the actin cytoskeleton. Ubiquitin has a molecular weight of about 8 kDa and is induced by heat, binding to damaged proteins. It is present constitutively, associated with histones in the chromatin of the nucleus. After induction by a stress, it binds to damaged proteins and facilitates their proteolytic destruction.

#### **The heat shock response in 9.5 day rat embryos**

Mirkes and colleagues at Seattle and Walsh and colleagues at Sydney extended the work by Cockroft and New, by using rat embryos in culture to study induced thermotolerance and the heat-shock response and its effects on post-implantation embryonic development.

Walsh et al. (1985, 1987) showed that exposure to 42°C for 10 min on day 9.5 resulted in normal, but slightly developmentally retarded embryos when examined at 11.5 days. This exposure also conferred a significant degree of protection against a subsequent exposure to 43°C for 7.5 min, which otherwise causes severe malformations. The acquisition of thermotolerance after the 42°C exposure required a recovery period of at least 15 min at



38.5°C. Thermotolerance was not acquired during continuous exposure to 42°C for 20-40 min, which caused severe head defects. The acquired thermoprotection persisted for at least 8 h and during this period general protein synthesis was reduced while synthesis of proteins of 71-73 kDa and 90 kDa increased (Fig. 2). Control embryos contained high levels of 73 kDa and 90 kDa proteins. Based on their molecular weights and appearance during the heat shock response, these proteins were assumed to be members of the HSP70 and 90 families.

Following these initial studies, the heat shock response was examined in more detail again using 9.5 day rat embryos given one of three types of exposure:

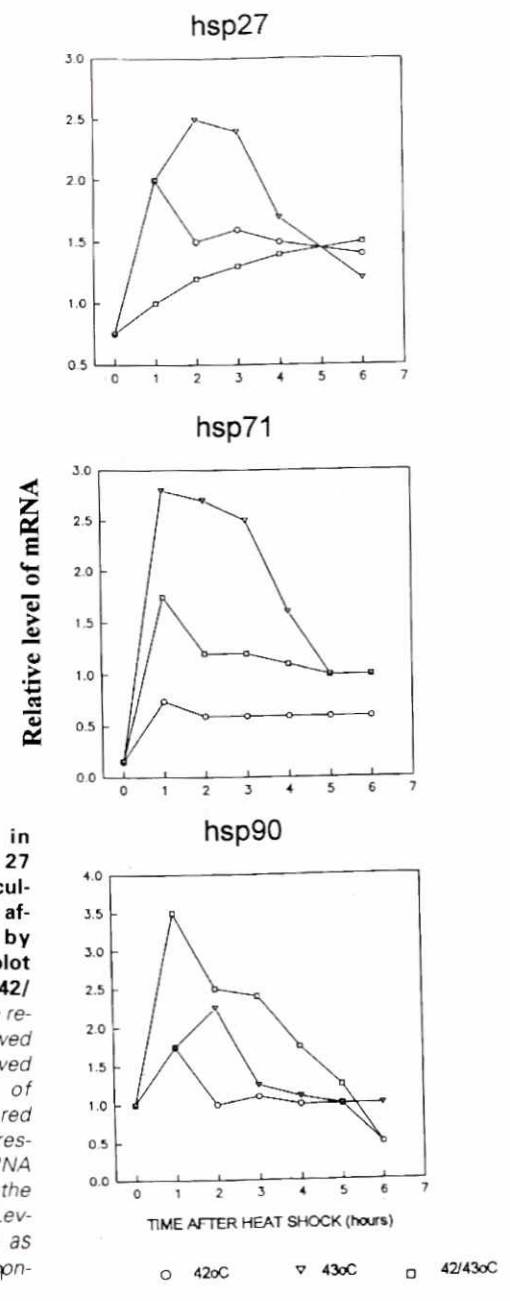
- 42°C for 10 min which does not cause defects, but following an adequate recovery period at 38.5°C, it confers thermoprotection against a subsequent damaging exposure;
- 42°C for 10 min, followed by recovery at 38.5°C for 1 h, then 43°C for 7.5 min. These embryos show no developmental damage at 11.5 days; or
- 43°C for 7.5 min which causes severe developmental damage (Fig. 3).

In these studies (Walsh *et al.*, 1989, 1991, 1993, 1994), HSP90, HSC73 and HSP23-27 were found in normally developing control 9.5 day rat embryos but HSP71 was not detected. At 1.5 h after the damaging 43°C exposure, there was a reduction in total protein synthesis of over 30% and at 2-3 h a four fold increase in the synthesis of HSP71/73 and a two fold increase in HSP90. The levels of synthesis of total protein and HSP71/73 and 90 had returned to normal by 6-8 h (Fig. 4).

Recently, the expression of HSP47 has been studied by Li (unpublished). Using immunohistochemistry in 7.5 day rat embryos, it was found to be confined to parietal endoderm cells. At 8.5 days it was present in ectoderm, mesoderm and endoderm. It was widespread on day 9.5 appearing generally through the ectoderm, mesoderm and endoderm of the embryo, and the allantois and yolk sac. After 9.5 days it was found in more regionally specific areas including the brain, branchial arches, heart and somites and except for ectodermal tissues, coinciding generally with the distribution patterns of collagens I and IV. Although HSP47 was found in the ectoderm at day 9.5, no collagen could be identified in it until after the commencement of neural tube closure.

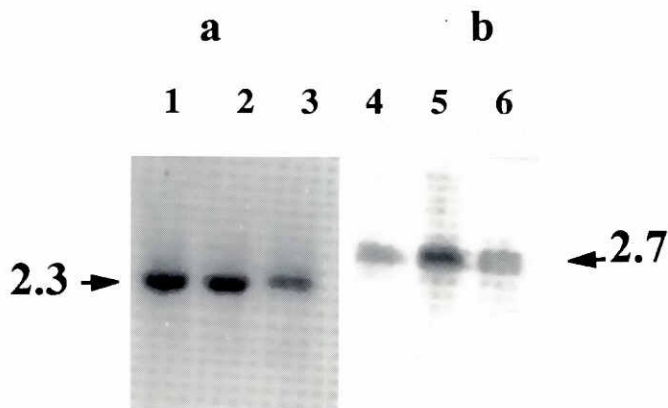
Changes during the heat shock response in levels of hsp27, 71 and 90 mRNA were studied using Northern (Fig. 5) and dot blot analysis. Northern analysis could not detect 71 kDa mRNA in control embryos but it was present in small quantities at 60 min after exposure to 43°C and reached a peak of about 8 fold over resting levels in 90 min declining to normal levels in 5 h. The levels in the 42/43°C embryos were much less than the 43°C group. HSC73 was present in all samples, in small amounts in controls and embryos exposed to 42°C but in larger quantities in embryos after the exposures at 43°C and 42/43°C (data not shown). HSP27 was also induced quickly by heat shock and after the 42°C exposure rose to about a two fold level between 0.5-3 h falling to normal levels by 6 h. Much higher expression occurred after the 43°C exposure and the 42/43°C treatment resulted in levels lower than both the 42°C and 43°C responses. The response of hsp90 mRNA was different. Following 42/43°C levels were higher than those for 43°C which in turn were higher than those for 42°C, suggesting that the response for hsp90 mRNA is proportional for the total dose of heat.

Dot blot analyses after 42°C exposure showed a rapid activation of hsp71. Within 20 min, a 2-3-fold increase of mRNA was detected in the nucleus, increasing slightly over the next 60 min and then falling to control levels in 3-4 h. The levels in cytosol showed a similar response but returned to normal within 2.5 h. Following the 43°C exposure, the nuclear mRNA also showed a 2.5-fold increase within 20 min, this level being maintained for over 4 h and falling to base levels. The cytosol fraction also showed a rapid increase to a 6 fold peak at 1.5 h, falling rapidly to normal levels by 5 h. The hsc73 mRNA showed about a 2 fold increase within 60 min. The thermotolerant embryos (42/43°C) showed an intermediate response.



**Fig. 5. Changes in hsp90, 71 and 27 mRNA in 9.5 day cultured rat embryos after heat shock, by Northern and dot blot analysis after 42°C, 42/43°C and 43°C.** The response to 42°C followed in 1 h by 43°C showed downregulation of hsp71 and 27 compared with 43°C. The expression of hsp90 mRNA was proportional to the total dose of heat. Levels are expressed as values relative to control levels.





**Fig. 6.** Constitutive expression of hsp47 mRNA, the band marked (arrow) at 2.3 kb (a) compared with expression of collagen type 1V mRNA distribution, (arrow) with band marked at 2.7 kb and (b) in 9-11 day cultured rat embryos. 10  $\mu$ g of total RNA was used per lane and separated by 1% agarose gel electrophoresis. (Lanes 1, 2 and 3) represent hsp47 mRNA on days 9.5, 10.5 and 11.5 respectively. (Lanes 4, 5 and 6) represent collagen on days 9.5, 10.5 and 11.5 respectively. Note the strong expression on days 9 and 10.

*In situ* hybridization studies of control 9.5 day embryos using hsp cDNA probes, showed constitutive expression of hsp71 only in the allantois and ectoplacental cone. In embryos given the 43°C exposure, maximum hsp71 expression was at 90-120 min in the neurectoderm of the neural plate, its underlying mesoderm and at low levels in the endoderm. Expression was most marked around the anterior neuropore. In 10.5 and 11.5 day embryos given the 43°C exposure, the overall hsp71 expression was reduced being greatest around the mid- and hindbrain areas.

The developmental expression and distribution of hsp47 mRNA was studied recently, using Northern blot analysis and whole mount *in situ* hybridization (Li, unpublished). With the exception of ectodermal structures, the expression of hsp47 mRNA coincided with the distribution patterns of collagens I and IV. Although hsp mRNA was expressed in the ectoderm before neural tube closure, collagen was not detected until after it commenced. It was expressed most strongly between days 9.5 and 11.5 (Fig. 6) and at lower levels at days 13.5 and 14.5 and was found in ectoderm and endoderm of 8.5 day embryos. It was widespread in the neural plate, yolk sac, allantois and chorion at 9.5 days. At 10.5 days its distribution was confined to the brain, heart, branchial arches and somites.

#### The heat shock response in 10.5 day rat embryos

Mirkes (1985, 1987) used day 10 (6-10 somite) rat embryos. Controls were cultured throughout at 37°C. A second group was at 37°C throughout except for 30 min at 42°C, given 1 h after the culture started which caused no apparent damage. A third group was given 43°C for 30 min at 2.5 h after commencement of culture and this caused a high mortality and level of defects. A fourth group received exposures of 42 and 43°C as above, separated by 1 h at 37°C and these embryos had partial protection against mortality and malformation.

Within 1 h, the 43°C treatment induced the synthesis of eight proteins of 28, 31.5, 33.5, 34, 39, 69, 78 and 82 kDa. Two proteins (33.5 and 34 kDa) were also detected in control embryos. Synthe-

sis of all induced proteins ceased between 3 and 9 h after exposure except 28 kDa which ceased at 1-3 h. Usually, normal protein synthesis is curtailed during the heat shock response but in these experiments, the 43°C embryos did not show such a reduction. After the 42°C exposure, synthesis of 31.5, 39 and 69 kDa proteins was induced and the combined treatment, except for the 28 kDa, induced all proteins at about half the level of the 43°C treatment.

Fisher *et al.* (1995) also used heat shock in day 10 rat embryos *in vivo* and *in vitro* to examine the role of the heat shock response in the genesis of defects of somite segmentation, resulting in vertebral and rib anomalies of the mid thoracic region in which the severity of damage to somites was related to the dose of heat (Cuff *et al.*, 1993). After exposures *in vivo* to 42-42.5°C for 5 min or *in vitro* to 42-42.5 for 15 min, they found enhanced synthesis of a 70 kDa protein for 1 to 4 h and of a 90 kDa protein for 1 to 8 h. Transcription was required for its expression. The 70 kDa protein was identified by Western blot as an inducible HSP72 which accumulated and remained in the neurectoderm 2-27 h after exposure. It was not detected in the somite mesoderm. There was a lag period of 18 h between accumulation of HSP72 and the appearance of abnormal segmentation. The authors commented that the absence of heat shock response in the somite mesoderm might explain its sensitivity to heat. However, this argument cannot be applied to the damage caused by heat on day 9 to the neurectoderm which accumulates large amounts of induced HSP before and during the period of abnormal neural plate development (Walsh *et al.*, 1987). In studies on somite segmentation, analysis of the hsp47 response might be rewarding.

Mirkes *et al.* (1991) compared the response in days 9 (pre-somite), 10 (6-10 somite), 11 (21-25 somite) and 12 (31-35 somite) rat embryos which were equilibrated for 1 h at 37°C, then given 43°C for 15-60 min and returned to 37°C for 1 h before processing. The HSP response was analyzed by two-dimensional gel electrophoresis and mRNA by Northern blot analysis. Day 9 embryos synthesized HSP of 28-78 kDa, and on day 10 essentially the same set with two additional HSP (34 and 82 kDa) were found. The response on day 11 was limited to 31, 39 and 69 kDa HSP and on day 12 none were found. Northern blot analysis of hsp70 mRNA showed a response which varied with the stage of development and the dose of heat. The day 9 embryos showed the greatest response which was increasingly attenuated from days 10 to 12, especially with the 60 min dose.

It is known that agents other than heat can elicit a stress response and Mirkes and Cornel (1992) compared the effects of one such agent, arsenic, with heat shock on 10 day rat embryos *in vitro*. After 1 h culture at 7°C, sodium arsenite was added to the culture medium to a level of 50  $\mu$ M which was removed after 2.5 or 5 h and normal culture conditions resumed for the remainder of the 24 hour total culture period. The heated embryos, after 1 h at 37°C, were given an exposure of 15 min at 43°C and the remainder of the total 24 hour culture period was at 37°C. All HSP families and hsp70 mRNA were measured. The sodium arsenite treated embryos synthesized a set of HSP which was indistinguishable from heat shock set and a monoclonal antibody to the inducible HSP72 identified it in embryos exposed to hyperthermia or sodium arsenite. Northern blot analysis for hsp70 mRNA revealed none in control embryos, minimally detectable levels after exposure to sodium arsenite for 2.5 h, relatively large amounts in embryos given 5 h of this treatment and similar amounts following hyperthermia. Although the response was similar, the defects caused by the



two treatments were different, hyperthermia causing more severe damage to the developing prosencephalon, rhombencephalon and eyes.

In another study (Mirkes and Doggett, 1992), a monoclonal antibody to HSP72 with Western blot analysis, identified this HSP in 10 day rat embryos exposed to temperatures exceeding 40°C for various periods. The smallest doses associated with synthesis were 41°C for 15 min, 42°C for 15 min and 43°C for 2.5 min. The smallest doses producing defects were, 41°C for between 45 and 60 min, 42°C for between 15 and 30 min and 43°C for between 0 and 2.5 min. HSP72 was detected at 2.5 h after exposure and persisted for 24-48 h, which raises the possibility of it being used as a biomarker for embryotoxicity or for a previous exposure conferring tolerance.

#### Studies with mice

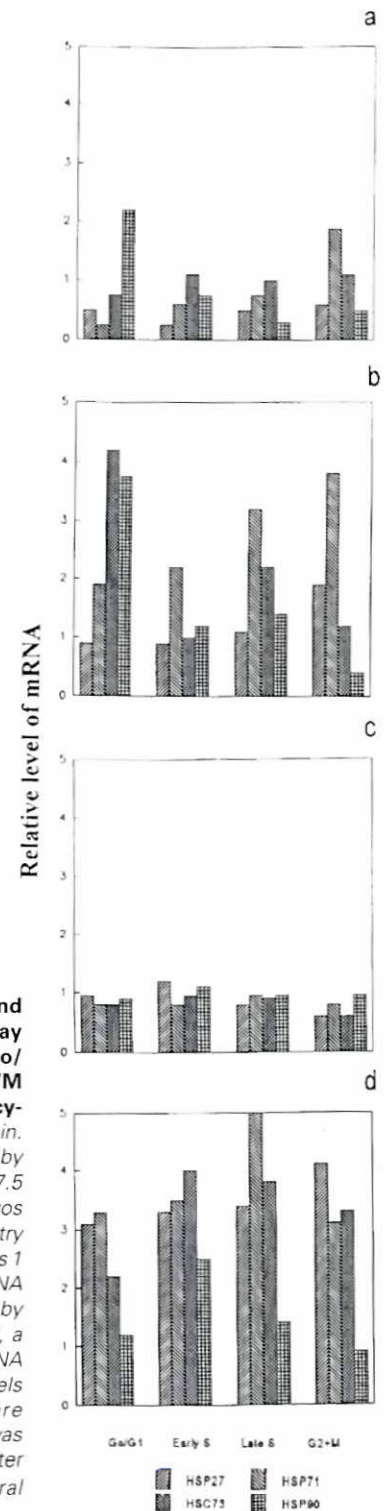
Kapron-Bras and Hales (1991) induced thermotolerance in 8 day (4-8 somite) CD-1 mouse embryos in culture by exposure to 43°C for 5 min followed by a 30 min recovery period at 37°C. The pretreated embryos were partially protected against exposure at 43°C for 20 min, which was toxic for unprotected embryos. Although it gave some protection against death and defects of branchial arches and turning, it did not protect against some defects such as small forebrain and microphthalmia while the incidence of exencephaly was increased. The pretreated embryos had better protection against death and malformation caused by a toxic level of cadmium. These results differ from those of Mirkes (1985, 1987) and Walsh *et al.* (1987) who used 10.5-day (6-10 somite) and 9.5-day (head fold, pre-somite) rat embryos respectively. Their pretreated embryos which were later given a toxic dose of heat were protected against growth retardation and malformation. The discrepancies between these mouse and rat studies could be due to differences in species response and in developmental stages at the time of exposure.

#### Comparisons and disparities between *in vitro* and *in vivo* studies

There is difficulty in interpreting and correlating much of the *in vitro* data and relating it to *in vivo* studies because of varying experimental conditions, including differences in the stage of embryonic development at exposure, in the doses of heat and in incubation temperatures of control embryos. Incubation temperatures for control embryos vary between 37 and 38.5°C. Our evidence indicates that the mean resting core temperature of rats is 38.5±0.5°C (Germain *et al.*, 1985) and this temperature is used in our normal 9.5 day rat embryo culture system (Walsh *et al.*, 1987), 42°C (+3.5°C over controls) for 10 min is used to induce thermotolerance and 43°C (+4.5°C) for 7.5 min to cause defects. Mirkes (1985, 1987), Mirkes *et al.* (1991) and Harris *et al.* (1991) cultured their control 10 day (6-10 somite) rat embryos at 37°C, using 42°C (+5°C) for 30 min to induce thermotolerance, 43°C (+6°C) for 30 min to cause defects or 43°C for 15, 30 or 60 min to study the heat shock response in 9-12 day embryos. G.L. Kimmel *et al.* (1993), used 10 day (10-12 somite) rat embryos, a control incubation temperature of 37-38°C and caused deficits in development of forelimbs with 10 min at 42-42.5°C (+4-5.5°C), and of forebrain, midbrain and optic system with 15 min at 42°C (+4-5°C).

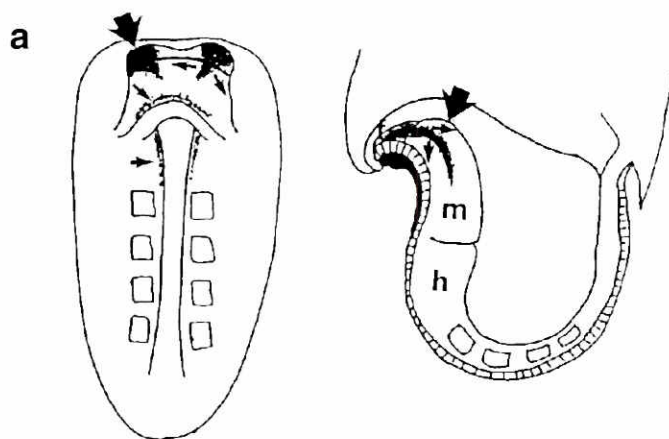
In the *in vivo* studies by Germain *et al.* (1985) using 9.5 day rat embryos, 42°C (+3.5°C) for 10 min caused microphthalmia in over one-third of the exposed embryos while 20 min caused abnormal

development in all surviving offspring. At 43°C (+4.5) for 10 min 94% of embryos died and at 20 min all died. Some of these disparities between *in vitro* and *in vivo* results are probably related to differences in the susceptibility to damage at the 9.5 and 10.5 day stages. Webster *et al.* (1985) found that heating pregnant rats on day 9 caused much more damage to the structures of the head

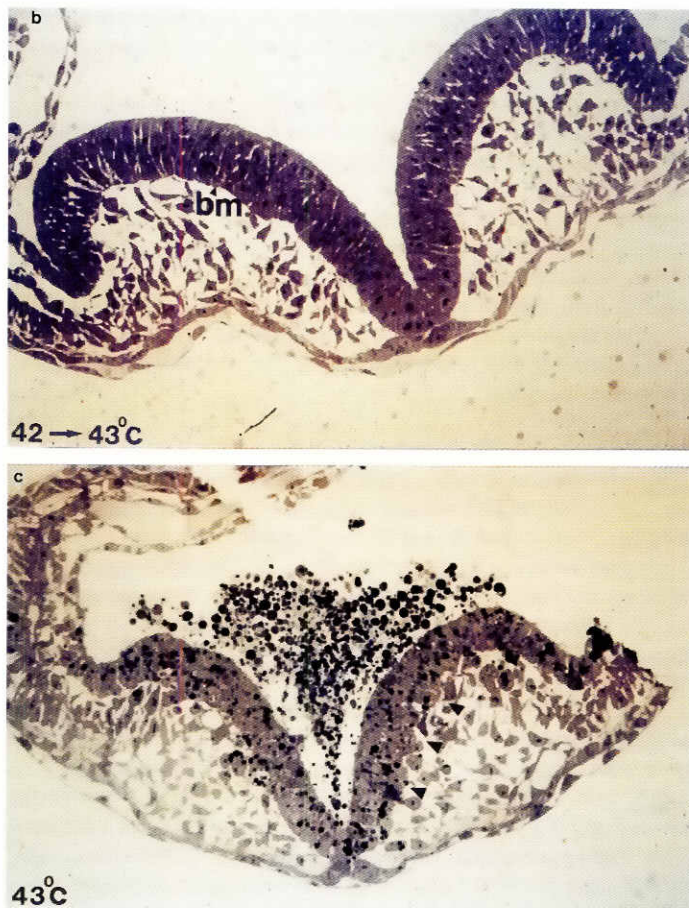


**Fig. 7. Changes in hsp90, 71 and 27 and hsc73 mRNA in 9.5 day cultured rat embryos during Go/G1, early S, late S and G2/M phases of the cell replication cycle. (a) Control. (b) 42°C for 10 min. (c) 42°C for 10 min followed by 38.5°C for 1 h, then 43°C for 7.5 min. (d) 43°C for 7.5 min. Embryos were processed by flow cytometry and dot blot and Northern analysis 1 h following treatment and mRNA was identified and measured by using cDNA probes. After 42°C, a four fold increase of all hsp mRNA occurred relative to control levels and at 43°C all hsp were "overexpressed" while hsp90 was severely depressed in Go/G1. After 42/43°C, there was a general downregulation of all hsp.**





**Fig. 8. Cell death in the neuroepithelium of 9.5 day cultured rat embryos following heat exposure.** (a) Line drawings of dorsal and lateral views of the neural plate at the 4 somite stage showing early differentiation into forebrain (f), midbrain (m) and hindbrain (h). Shaded areas (arrows) are most severely affected by cell death following heat shock. (b) Transverse section through the anterior region of the neural plate 5 h after exposure to 42°C for 10 min followed by 60 min at 38.5°C then 43°C for 7.5 min showing protection against heat damage. (c) Transverse section of the neural plate 5 h following exposure to 43°C for 7.5 min, showing severe disruption of the basement membrane (arrows) and extensive death of neural plate cells many of which have spilled into the amnion.



than on day 10 which suggests that the heat shock response in the head would be different on day 9.5 than on day 10.5. Some of the disparities could also be due to differences in the dose of heat delivered to embryos in the two systems. Also, it should be kept in mind that exposures of 43°C for 7.5 to 60 min as given *in vitro* would cause prenatal death of nearly all non-thermotolerant 9.5 day embryos *in vivo*. The analysis of the heat shock response *in vitro* is usually carried out a few hours after exposure. Although it might be alive at the time of analysis following such extreme doses, the results might be a measure of the maximum response of a moribund embryo.

#### Developmental regulation of HSP expression

As some constitutive HSP are present at certain embryonic stages, it can be inferred that they play a role in normal development. The 70 kDa family are the first to be expressed constitutively at the 2 cell stage and HSC70 is also at high levels at the 8 cell stage (Mezger *et al.*, 1991). In the mouse, very active constitutive expression of members of the 90 kDa, 70 kDa and 60 kDa families occurs at the 8 cell stage, with the heat inducible HSP86 and HSC84 and 70 being most prominent. During subsequent development, this level of synthesis is maintained. These constitutive stress proteins form a relatively large proportion of the total protein content of the neuroectoderm at day 8, the stage of neural induction in the mouse (Mezger *et al.*, 1991) and in the rat, at days 9.5-11.5, the stages of neural induction and major organogenesis (Walsh *et al.*, 1989). Transcriptional activation is mediated by a heat shock

transcriptional factor which is present in the cytoplasm and nucleus of unstressed embryonic cells. At some stages of development and in response to heat or other stresses, the transcription factor accumulates in the nucleus, binding to DNA at a site known as the heat shock element (Morimoto, 1993).

The inducible HSP are not found normally in embryonic tissues except in small amounts as they are not transcribed, but their synthesis can be induced by heat at certain stages of development. The heat inducible hsp70 can be induced for a brief period after cleavage to form a 2 cell embryo and is strongly induced after the blastocyst stage (Morange *et al.*, 1984; Heikkila *et al.*, 1985). It is not inducible in mouse and rabbit embryos between the 2 cell and blastocyst stages. The work outlined above indicates that the response can be induced strongly during the major stage of organogenesis in the rat (days 9-12). With the known functions of the HSP, and the evidence that the onset, and the duration of inducibility of hsp coincides with onset and duration of the most critical stage of organogenesis, it appears that a function of the heat shock response could be to provide protection against embryonic damage by heat and other stresses at vulnerable stages of development.

Using flow cytometry, Walsh and Morris (1989) and Walsh *et al.* (1994) studied the levels of hsp27, 71, 73 and 88 (90) mRNA at various stages of the neuroectodermal cell generation cycle and the effects of the various heat shock regimes (Fig. 7). The analysis was done at 1 h after heat treatment. In control 9.5 day rat neuroectoderm, there was a steady expression of hsc73 throughout the cycle and



high expression of hsp90 at Go-G1 and of hsp 71 at G2-M. With 42°C for 10 min, the levels of all mRNA were elevated in Go-G1 and late S phases, with high levels of hsc73 and hsp90 at Go-G1, while hsp27 and 71 were elevated above control levels at all phases, particularly at late S and G2-M. With 43°C for 7.5 min, hsp27 expression was greater throughout the cycle than for any other treatment, hsp71 was strongly expressed in late S and hsc73 was elevated in S and G2-M phases while hsp90 was severely depressed in G0/G1. It could be argued that the different levels of expression of the hsp at the various phases might reflect a response to different levels of damage to cell constituents but this proposal has not been tested. Some weight is given to this interpretation by the response following the combined, non teratogenic treatment of 42°C followed by 43°C in which there is a general downregulation of expression of all hsp compared with the expression after either single treatment.

#### Cell death after exposure to hyperthermia

In the studies by Cockroft and New (1978), histological examination of embryos at 40.5°C and 41°C over the 48 h culture period showed widespread cell death, particularly in the brain and spinal cord. In embryos cultured at 41°C parts of the nervous system were entirely necrotic. Neuroepithelial cell death was also the prominent feature of the pathological findings in heated guinea pigs (Edwards *et al.*, 1974; Wanner *et al.*, 1976; Upfold *et al.*, 1989), mouse (Shiota, 1988) and rat embryos *in vivo* (Skreb and Frank, 1963; Harding and Edwards, 1993), and *in vitro* (Cockroft and New 1978; Mirkes, 1985; Walsh *et al.*, 1987, 1991, 1993) and appeared to be the most important basic mechanism underlying the neural defects caused by heat (Fig. 8). Cell death, usually of less severity, is also observed in mesodermal cells, but is very infrequent in endodermal cells. In guinea pigs, mitotic cells of the 21 day embryonic neuroepithelium are the most sensitive, abnormally clumped chromosomes being found immediately after a 45 min spike elevation of approximately 2°C. Also after 4-8 h, cells presumed to be in S-phase and some mesoderm cells showed apoptotic cell death. Heat damage to cells in S-phase requires an elevation of at least 3-3.5°C. During the 4-8 h period, normal mitotic activity ceases and this period of inhibition is followed by a synchronized burst of mitotic activity (Edwards *et al.*, 1974; Wanner *et al.*, 1976; Upfold *et al.*, 1989). The damaged M and S phase cells break up and are removed within a few to 24 hours. Walsh *et al.* (1991, 1993) showed in 9.5 day cultured rat embryos, that 42°C (+3.5°C) for 10 min caused death of a small number of cells in the G2 or mitotic phases of the cell cycle at 5 h and also at 12-15 h after exposure, possibly after a division had occurred (Fig. 9). No developmental abnormalities result from this dose of heat. However, a teratogenic exposure of 43°C (+4.5°C) for 7.5 min, was followed at 3-5 h by very extensive apoptotic cell death in the neuroectoderm particularly in the ridges of the neural folds (Fig. 8). This exposure *in vivo* causes microphthalmia in 100% of surviving embryos (Germain *et al.*, 1985). In their flow cytometry studies, Walsh and Morris (1989) also found that the heat stressed neuroectodermal cells caused a partial synchronization of the generation cycle by causing cells to accumulate for 1-2 h at the G1-S boundaries after treatments of 42°C, 43°C or 42/43°C. Following treatments of 42°C or 42/43°C there was also a barrier to cell progression at the S-G2 boundary. Using squash preparations from heated 21 day guinea pig embryos, Edwards *et al.* (1974) found blocks to progression through mitosis before prophase and during metaphase.

The apoptotic cell death in embryos damaged by heat at neurulation should not be confused with the apoptosis which determines the shape of a structure or deletes supernumerary cells as a normal embryological mechanism (Glucksmann, 1951). At the time of neurulation, very few apoptotic cells are normally found and never in the very large numbers which follow a damaging exposure to heat. It has been suggested that once cells are committed to commence a division cycle, apoptosis is initiated and when the cell reaches check points with acceptable progression in the cell cycle it passes to the next phase (Kung *et al.*, 1990). Sufficient heat damage to the functional proteins of dividing embryonic cells might well prevent the successful completion of the immediate or the subsequent cycle and allow the apoptotic program to be implemented.

#### Discussion and conclusions

The information yielded from the *in vivo* and *in vitro* studies allows some conclusions and speculations to be made. A fundamental question is, what makes some embryonic cells extremely sensitive to damage by heat and other toxins? Embryos can tolerate variable doses of heat, the dose causing damage depending mainly on the stage of development. The most clearly defined threshold elevation of temperature causing defects *in vivo* is 2-2.5°C, in the form of a spike over a total heating and cooling period of about 80 min in 21 day guinea pig embryos (Edwards, 1969b) and as a plateau elevation of 2.5°C for 60 min in 9.5 day rat embryos (Germain *et al.*, 1985). A similar threshold temperature elevation has been shown for 9.5 day rat embryos in culture (Cockroft and New, 1978). It has been suggested that the threshold of 2-2.5°C exists because of the presence in cells of constitutive, heat shock 90, 70, 47 and 27 chaperone proteins which can protect against denaturation (Edwards *et al.*, 1995) so the threshold might represent a quantitative measure of the denaturation required to titrate out the constitutive proteins. It has long been known that heat denatures proteins (Mirsky and Pauling, 1936) and even at normal body temperatures (37-40°C), proteins are denatured. At 37°C,

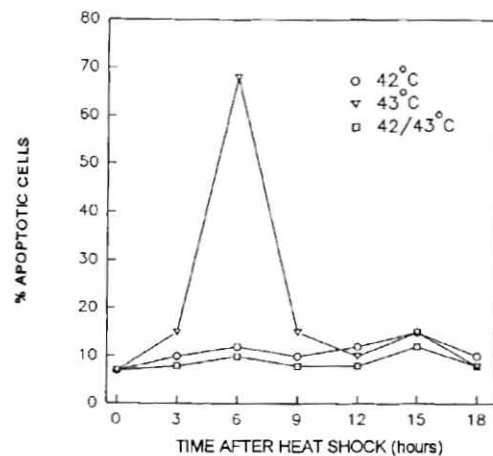


Fig. 9. Cell death expressed as a percentage of all cells in the anterior neuropore region of the neuroectoderm of 9.5 day cultured rat embryos following 42°C for 10 min, 43°C for 7.5 min and 42/43°C treatments. Note the very high level of cell death at 4-8 h after 43°C and the small second wave of deaths occurring some hours later in each treatment.



this results in an estimated loss of over 0.2% of cells per hour (Johnson and Pavelec, 1972).

It has been proposed that the heat shock response takes precedence over other normal developmental events, altering the established programs of gene activity and resulting in defects and that the response can be elicited by diverse environmental agents which act along a common pathway producing similar defects at similar stages of development (German, 1984). There is ample evidence that a strongly protective heat shock response need not be teratogenic, but there is equally strong evidence that a teratogenic dose of heat is associated with highly elevated hsp mRNA and HSP and that cell death is a prominent feature of the damage to the nervous system (Walsh *et al.*, 1989). Perhaps there is a threshold level for the response, above which German's hypothesis applies, and below which apparently complete recovery occurs. If cells die because changed gene activity during the heat shock response leads to apoptosis, the hypothesis is sustainable. The basic question is whether defects occur because of the activity of the heat shock response or because of its failure to protect.

Close examination of the stages at which most damage is caused to embryos indicates that it is at the inductive stage for the formation of an organ (Edwards *et al.*, 1995). The stage of active cellular proliferation to form the organ is less susceptible to damage by the same amount of heat and after formation, the organ becomes relatively resistant.

From the evidence that the heat shock response is greatest in organs most susceptible to heat, that the heat shock response can fail to protect embryos during the inductive phase of organogenesis and that cells which die are concentrated in the immediate region being induced (Fig. 8), it is possible to suggest some mechanisms behind the production of defects. One possibility is that during the induction of the organ, the gene activity might be committed largely to that activity, resulting in a delay or reduced efficiency in induction of a protective heat shock response. It is also possible that during the induction of an organ, the protective, constitutive heat shock proteins are largely requisitioned by heat susceptible, newly synthesized proteins and are unavailable as a reserve. A third possibility is that the cells partially induced for organogenesis cannot mount a heat shock response and survive. The next question needing resolution is why the heat damaged embryo is unable to replicate more cells to make up the mass required to form a normal organ? In many instances it appears that one further division by the replicating cell population would easily make up the deficit. For instance, after heat damage to an embryo at neural tube closure, the compensatory proliferation of only a minor proportion of the total cell population could provide the mass required for closure. Similarly, the first, rapid phase of neurogenesis of the fetal guinea pig brain ceases at days 51-53 whether the target growth has been achieved or not (Edwards, 1981) and a possible explanation for a "timed" termination of proliferation is that, at induction of an organ, the induced cells are programmed to a finite number of divisions (Edwards *et al.*, 1976). In this model, cells lost by apoptosis following heat exposure would not be replaced. The resolution of these uncertainties is likely to come from studies using embryos in culture.

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