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Short Contribution

Hyperthermia in the chick embryo: HSP and possible mechanisms of developmental defects

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ABSTRACT Although hyperthermia is an established teratogen in all species studied and the cellular heat shock response is well known, the mechanisms of developmental deviation remain obscure. We have used a chick model system in which fertilized eggs containing embryos at presomite and/or early somite stages (HH 4-10) were exposed to 45°C for 180 min. Six hours following treatment we did not observe any overt morphological disturbance, but at twelve hours following exposure (when controls reached HH 11-13) embryos exposed at late streak stages (HH 4-6) exhibited severe malformation of the head. Embryos exposed later (HH 6-9) manifested spina bifida at the thoracic and lumbosacral levels. Mirror image heart looping was also observed in 20% of these embryos. Paraxial mesoderm was apparently unaffected. Changes in cell proliferation and induced cell death preceded morphological changes. We used acridine orange and confocal laser microscopy to demonstrate that hyperthermia induced cell death in neural folds starting 6 h following treatment. To assess cell proliferation, we used BrdU incorporation for 4 h. Immunodetection on paraffin sections demonstrated that proliferation was inhibited 6 h after treatment. Heat-exposed embryos exhibited the heat shock response, with protein expression reaching a maximum 4-6 h following heat treatment. Malformed embryos showed an intense heat shock response for a further 6 h. The levels of induced heat shock proteins were similar in the affected neural tube and in the heart, where neither induced cell death nor malformations were observed.

KEY WORDS: *hyperthermia, chick embryo, HSP, teratogenesis*

Dysmorphology

Hyperthermia (that is 43°+/-0.5°C for the last 30 min of a 3h incubation, confirmed by thermistor probe inside the egg) affected the neural tube development (70% of exposed embryos) and heart loop position (20%) in the chick embryo. The localization of defects depended on the stage of development. Twelve hours following heat treatment of embryos at presomite stages (HH 4-6) anterior neural tube defects (truncated head or unclosed neural tube) were most common. Embryos with a truncated head had no optic vesicles, and the primary divisions of the brain were absent. In the most severe cases, the remnant of the anterior neural tube was bent. Embryos with unclosed neural tube had defects in the area of the rhombencephalon or myelencephalon. Centrally positioned (ventral) or inverted heart loops were frequently seen in these embryos.

Embryos treated at early somite stages (HH 7-9) developed spina bifida defects located mainly in the posterior part of the neural tube. Hypoplastic brain vesicle were observed in only 20% of embryos. Abnormal position of the heart loop was seen in 15% of embryos, and inverted body turning in 8%. Lethality was not increased immediately after treatment nor in the following twenty-four hours. The caudal part of the trunk developed normally and paraxial mesoderm was unaffected by the treatment. With the exception of two embryos (adjacent somites) we did not observe any defects of somitogenesis, unlike more severe heat treatment (Primmet *et al.*, 1988). Untreated embryos used as controls did not exhibit any of the abnormalities described above.

Cell death

We found many dead cells in the anterior neural folds of HH 8 treated embryos (Fig. 1A). In embryos , treated at HH 7-9, widely open neural folds with an accumulation of acridine orange (AO) stained cells were found (Fig. 1C). Induced cell death seemed to be fully developed by 12 h of incubation at normothermic conditions

Abbreviations used in this paper: HSP, Heat shock protein; HH, Hamburger-Hamilton stage of development; BrdU, Bromodeoxyurine; PBS, Phosphate-buffered saline; TESPA, 3-triethoxysilylpropylamine.

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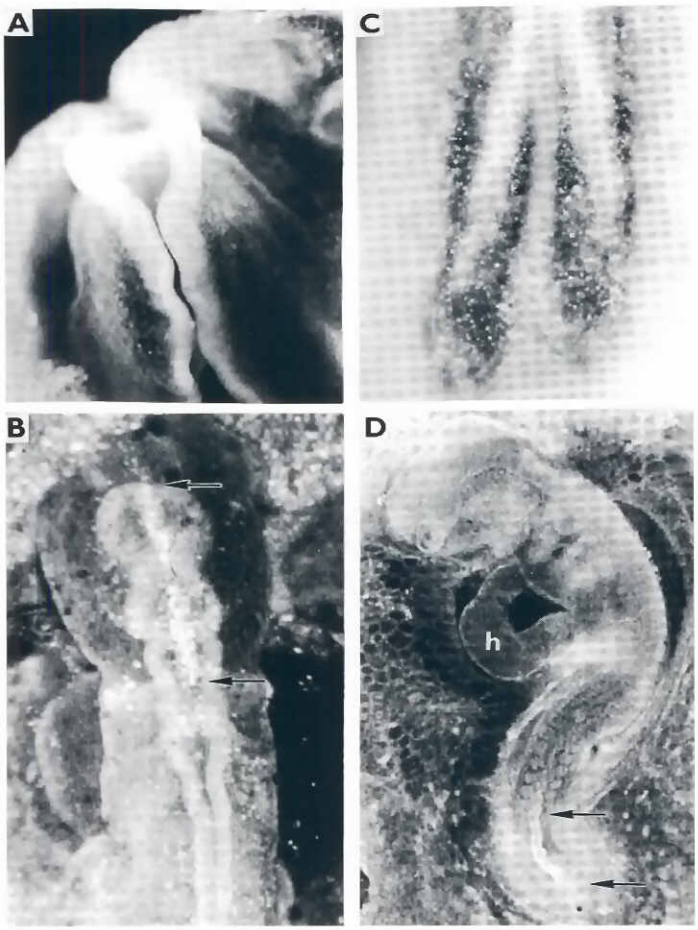

Fig. 1. Cell death pattern following heat treatment. (A) *Anterior part of 4-somite embryo (HH 8), estimated HH 4-6. at treatment. Six hours after treatment the neural folds are pervaded by apoptotic cells.* **(B)** *3D reconstruction of apoptotic cells (51 sections a' 25 μm) in the anterior part of embryo exposed as in A, but 12 h after treatment. The hindbrain area of dead cells present in normal embryos is substantially extended (arrows).* **(C)** *Caudal region of HH 10 embryo, estimated HH 7-9 at treatment. Six hours after treatment the neural folds are widely open. All structures are pervaded by apoptotic cells.* **(D)** *Whole embryo (HH14) exposed as in C, 12 h after treatment. The caudal part of the embryo is severely reduced and formed mostly of degenerating cells (arrows). h, heart.*

following heat treatment. Using confocal laser microscopy, we found that the areas of heat-induced cell death represent an anterior extension of a normal zone of programmed cell death (PCD) observed in the rhombencephalon (Fig. 1B). Somite stages (HH7-9) treated embryos had distinct area of cell death in the tail (Fig. 1D).

Dead cells were also detected in early neural derivatives (i.e., optic vesicles) 12h following exposure. No dead cells were found in the heart of treated embryos (see Fig. 1C).

Results of vital staining of the apoptotic cells was confirmed by hematoxylin staining of pycnotic cells in the neural plate of truncated head embryos (in paraffin sections).

Cell proliferation

In the neural plate of control embryos (HH 9), and in the paraxial mesoderm 6 h following treatment 80% of cells were proliferating as indicated by BrdU incorporation. (Fig. 2A). The proportion of proliferating cells was clearly decreased in the neural plate of treated embryo (HH 9) at similar levels of sectioning (Fig. 2B). Quantitative evaluation showed a 30% decrease in the number of labeled cells, whilst, the total cell counts did not differ between treated and untreated embryos.

In control embryos at HH 11-13 (equivalent to 12 h) the typical proliferation pattern showed labeled nuclei located predominantly within the basal plate (Fig. 2C). The proportion of labeled cells was not uniform throughout the embryo. We found the highest proliferative activity (100%) in the anteriormost area of the head. Another highly proliferative area was in the caudal part of the tail, behind the caudal neuropore. Here, we found a 40% decrease of proliferation in treated embryos.

Immunohistochemistry

Constitutively expressed HSP70 protein was localized in the cytoplasm of neuroepithelial cells (Fig. 3A). Constitutive expression was also found in the centre of somite and in the yolk sac. Localization of induced proteins was largely nuclear from four to six hours following exposure (Fig. 3B). There were no differences in the intensity of staining throughout the embryo. The intensity of cytoplasmic and nuclear staining in treated malformed embryos was even further increased at 12 h of incubation under normothermic conditions (Fig. 3C). However, the persisting heat shock response was not limited to the damaged part of the neural tube (see Fig. 3D). Intensive nuclear staining was also found in heart tissue, where no signs of injury were present. All examined

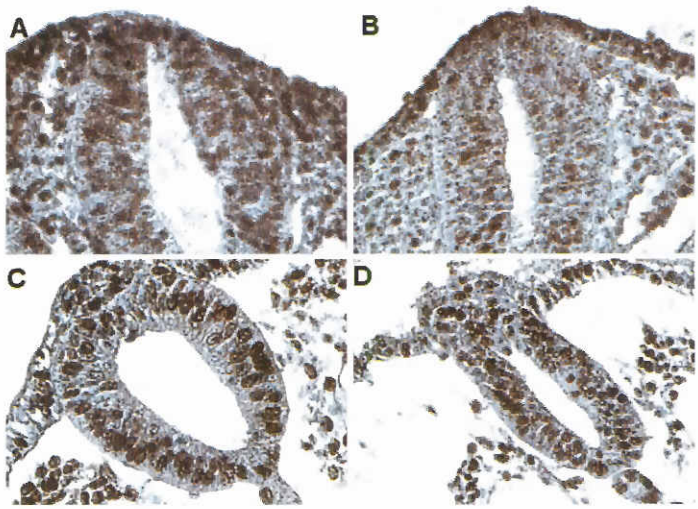

Fig. 2. Distribution of BrdU labeled cells. (A) *Transverse section through the somitic region of control embryo staged HH9. Numerous mitotic figures in the neuroepithelium are distributed around the well preserved circumference of the central canal. DNA synthesising elements (brown) constitute the majority of cell in the normal neural tube.* **(B)** *Transverse section through the somitic region of treated embryo staged HH8+. DNA synthesising cells are sparse.* **(C)** *Transverse section through the spinal cord (lower part). Normal proliferating structure with distinct distribution of heavy stained nuclei located in the germinal zone.* **(D)** *Slightly oblique transverse section through the spinal cord. The inner row of labeled nuclei is found in the immediate neighborhood of the internal limiting membrane. A huge mass of neural crest cells invades the lumen.*

embryos with clear morphological abnormalities induced by heat treatment (5 analyzed) expressed induced HSP in the increased manner, while apparently normal embryos revealed only cytoplasmic staining slightly higher than controls (not shown). Twenty four hours following treatment we observed only cytoplasmic staining of induced proteins in the whole extent of the neural tube in malformed embryos.

The first aim of this work was to find a regimen for reproducible induction neurulation defects in the chick. We found that heat treatment of presomite stage embryos was often followed by truncation of the head similar to vertebrate models (cultured rat embryos) treated in comparable developmental stages. In addition, in the chick hyperthermia disturbed the process of heart looping. This phenomenon was also reported in cultured rat embryos (Walsh *et al.*, 1997). In contrast, neurulation defects (unclosed neural tube and spina bifida) induced by heat treatment were not observed in mammalian embryos .

Six hours following treatment the occurrence of cell death coincided with both the site and severity of morphological defects. Accumulation of dead cells in four somite chick embryos was limited to neural folds in the area of presumptive head region. Previous studies in the rat also showed multiple apoptotic cells in the anterior neural folds of the heat treated 4 somite embryos (Edwards *et al.*, 1997).

Six hours following treatment we observed besides the onset of apoptosis, a clear cut inhibition of proliferation and/or a rapid decrease in amounts of S phase cells. The decrease of labeled cells observed in mesoderm was less severe than in the neuroectoderm. The observation that mitotic activity ceases during 4-8 h following heat treatment has been made in embryos of other species (Walsh *et al.*, 1997) . Our analysis of cell proliferation showed decreased numbers of cells in the neuroectoderm, compared to controls. In contrast, the total cell counts were unchanged at 6th h following treatment. Moreover, there were no pycnotic nuclei, nor mitotic figures observed in sections from treated embryos. Cell cycle arrest is one of the mechanism used by cells in response to damage. It may be the result of increased expression and accumulation of the p53 transcriptional factors known to trigger apoptosis (Levine 1997).

Both constitutively and inducibly expressed HSP70 RNAs are products of one gene in the chick (Morimoto *et al.*, 1986) unlike other vertebrates, where multigene HSP families are known. For example, constitutive and inducible HSP 70 RNAs are the products of different genes as shown in the rat (Thayer and Mirkes 1997). Whether this affects the developmental response to heat is not known. In our studies, the induced HSP 70 proteins appeared within 4-6 h after treatment and persisted 12-24 h in malformed embryos. Amounts of accumulated proteins are thought to be proportional to amounts of damaged proteins in the tissue. However, we observed comparable levels of heat induced HSP 70 proteins in damaged neural tube as in the heart, where no signs of induced cell death or tissue damage appeared. Interestingly, heat treatment used in this study affected only the direction of looping of the heart tube. We did not observe any other heart defects after heat treatment HH 4-9 chick embryos. On the contrary, frequent malformations (100% incidence of double outlet right ventricle) of the heart were observed on day 9, after hyperthermic treatment during the first 24 h of incubation (44°C).

A final question, the differences in the intensity of response of individual embryos. In our experiments, about 30% of treated

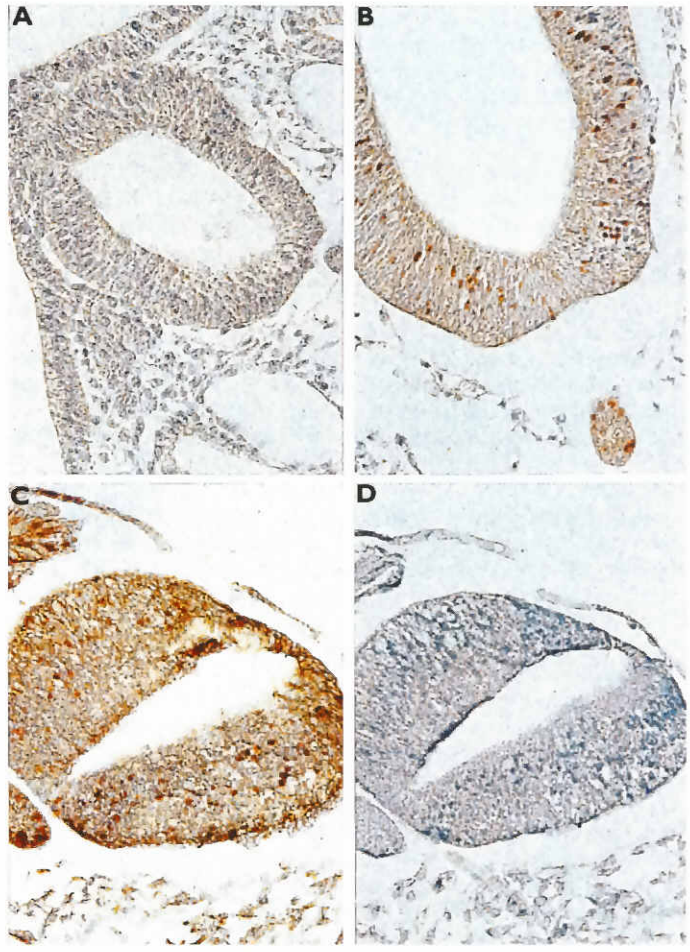

Fig. 3. Immunohistochemistry with anti HSP 70 protein. *All sections are transverse and counterstained with hematoxylin.* **(A)** *Control embryo HH 11 with constitutively expressed HSP.* **(B)** *Pattern of antibody labeled cells at the time of maximal response, 4-6 h following exposure.* **(C)** *The heavy staining of cells persists even 12h after exposure in malformed embryos.* **(D)** *Specific staining is absent in sections incubated without primary antibody.*

embryos did not seem overtly affected by heat treatment. If all embryos mount the heat shock response, as our data supports , then the question arises whether defects are induced because of hyperproduction of heat shock proteins, or because of insufficient response. This question is linked to the intriguing general problem of embryonic response to a harmful stimulus and the capacity for repair. Why cells lost by apoptosis are not replaced can only be answered by detailed understanding of the processes that control the regulation of cell population size during development.

Experimental Procedures

Fresh laid eggs of random-bred of White Leghorn Fowl (farm Dominant, Dobřenice) were used in all experiments. Under normal conditions, the eggs were incubated in a thermostatic oven at 37.5°C and 40-50% relative humidity.

Determination of hyperthermic regimen

Using a thermistor probe placed onto the surface of the embryo we monitored, at 15 minutes intervals, temperature changes within fertilized

eggs transferred into a 45°C incubator (humidity about 60%). After 150 minutes of incubation the temperature reached 43°C. A subsequent 30 min exposure of the embryo to temperatures over 43 and less than 44°C appeared sufficient for both the induction of HSP and dysmorphogenesis in 1.5 day-old chick embryos.

Treatment of closed eggs

Fertilized eggs were incubated at 37.5°C for 25 or 36 h in order to obtain embryos in stages HH 4-6 and HH 7-9, respectively. The Hamburger and Hamilton (1992) system was used. Distribution of stages was verified before each experiment. Ten embryos were staged *in ovo* by cutting a window in the egg shell, injecting 0.5% neutral red (in saline) over the embryo and assessed under a dissecting microscope. These embryos were discarded. Groups of about twenty eggs were exposed to hyperthermia 45°C for three hours and then returned back to normothermic conditions. A total of 104 embryos treated at presomite stages (HH 4-6), and 84 treated at HH 7-9, were used with the similar number of controls.

Cell death

Dissected embryos were stained 5 min at 37°C with acridine orange 5 mg/ml (Sigma Chemical Co,. Praha) freshly diluted from stock solution by Knudsen (1994), rinsed in HBSS (Hank's balanced solution) and examined in a fluorescent or confocal microscope (CLSM, MRC 600, Bio-Rad Microscience Ltd.).

Cell proliferation

We used the thymidine analog, bromodeoxyuridine, which is incorporated during S phase, to label proliferating cells. Seventy-five ml of 0,1M BrdU in PBS was dropped onto embryos at appropriate times following exposure, which were followed by further incubation at 37.5°C for 4 h before fixation (Henrique *et al.*, 1995). After exposure to BrdU, embryos were removed from eggs, washed in saline, fixed in Serra, then transferred into 70% ethanol and embedded in paraffin. Five mm paraffin sections were processed for BrdU immunodetection as described by Bellomo *et al.* (1996). Image analysis system (Lucia, Laboratory Imaging) was used for quantitative assessment of measurements of the fraction of cells in S phase.

Immunohistochemistry

Groups of 50 control embryos HH 7-21 (at least three for each stage) and 45 of exposed embryos at designated time points were used for this study. Embryos were rinsed in PBS, and placed in Serra's fixative overnight, embedded in paraffin and sectioned at 5 μm. Glass slide (TESPA coated) mounted sections were deparaffinized in Histoclear and placed in several changes of 100% ethanol. Further procedures were according to Thayer and Mirkes (1997). A monoclonal antibody SPA 822 (Stressgen Biotechnologies Corp., Victoria, Canada) in dilution 1:250 and biotinylated mouse IgG (Amersham, UK) (1:200) and Vector ABC kit (Vector Laboratories, Burlingame, CA) with DAB substrate (Sigma-Aldrich, Prague, Czech Republic) were used for immunohistochemical detection of HSP 70 proteins in transverse sections. Mayer's hematoxylin was used for counterstaining.

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