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Effect of Environmental Heat Stress on Embryonic Bone Development

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Effect of Environmental Heat Stress on Embryonic Bone Development

A Thesis

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By

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SUMMARY

SUMMARY

Birth defects are anatomical abnormalities present at birth. The causes of birth defects are genetic, environmental and multifactorial inheritance factors. The main objective of the thesis is to determine the effects of maternal heat stress during rat pregnancy on fetal axial skeletal development and to explore some of the possible maternal and placental responses to heat stress.

Experiment one: A group of 40 Wistar pregnant rats were randomly assigned to two treatment groups, a control group (non-stressed, n = 10) heat at 21^{0}C and a heat-stressed group kept at 41^{0}C (n = 30) for one hour on day 9 of gestation. The objective of this experiment is to determine the effect of heat stress on some maternal physiological parameters. Following an hour of heat stress or sham treatment, blood samples were collected from orbital vein, allowed to clot, and centrifuged at 3000 r.p.m for 10 minutes to obtain serum. Serum samples were used for determination of glucose, calcium, osteocalcin, thyroxin (T_4) and triiodothyronine (T_3).

Results showed that heat stress caused significant increases in serum glucose and oseocalcin levels. In addition, serum calcium, T_3, and T_4 levels were significantly lower in treated animals than those in control group.

Experiment two: A total of 34 Wistar pregnant rats were randomly assigned to three treatment groups, a control group (non-stressed, n = 10) kept at 21^{0}C, a heat-stressed group I kept at 41^0 C (n = 14), and a heat-stressed group II kept at 42^{0}C (n = 10) for one hour on day 9 of gestation. The objective of this experiment is to investigate the effect of heat stress on embryonic bone development and to demonstrate the extreme changes and severity of skeletal malformations due to temperature.

Results showed that heat stress caused reduction in the implantation, number of live embryos and fetal and placental weights in comparison to control animals. These effects were significantly pronounced in the 42^{0}C treatment group. Morphological malformations were found in fetuses due to heat treatment. Malformations in the upper and lower jaws and increased incidence of mandibular and maxillary hypoplasia were observed in heat-treated group as compared to the controls. But in comparison between 41^0 C and 42^0 C, the 41^0 C group showed a higher incidence of maxillary-mandibular hypoplasia and tongue protrusion. In addition, both experimental groups showed a high

incidence of excencephaly, exopthamia with cataract, facial clefts, and short tails than the controls.

More skeletal malformations were recorded in experimental animals than in controls. The control fetuses had well ossified bones of the skull that included the mandible, premaxilla, maxilla, zygomatic, nasal, frontal, parietal, interparietal, supra-occipital, exoccipital, temporal, tympanic ring, hyoid, ethmoid, presphenoid, basisphenoid and basioccipital bones than in the experimental animals. The comparison between 41° C and 42° C groups showed different responses in terms of skeletal defects.

Also results showed that control vertebral column appeared to have higher ossified vertebra than experimental groups. In the experimental fetuses, the vertebral arches and bodies showed decrease in number and poor ossification. The higher the temperature, the higher was the reduction in number of lumbar, sacral and coccygeal arches and bodies in experimental groups.

In the control group, ribs, and sternebare appeared normally ossified without any reduction in number. No instance of fused or hypoplastic ribs was found in control fetuses. There was lower incidence of hypoplastic ilium, ishchium and pubis in control fetuses than in experimental rats. In addition, higher development in forelimb skeletons was observed in the control animals than in the treated ones.

Placentas of the control groups showed lower weight compared with the experimental groups. The deciduas of experimental group was thicker than that of control group. There were large areas of hyalinization and lymphatic infiltration. Multinucleated giant cells were more abundant than those with single nucleus and basophils were extremely numerous. The glycogen cell clusters were reduced or absent over a large proportion of the spongy zone. Electron microscopic examination of the placentas showed a series of degenerative changes in experimental groups higher than those in control group placentas.

CHAPTER I

INTRODUCTION AND OBJECTIVES

INTRODUCTION

Birth defects are anatomical abnormalities present at birth. They are often referred to as congenital anomalies. They also include functional growth retardation disorders and inborn errors of metabolism all of which are of major clinical significance. Teratology is the branch of science that deals with the causes, mechanism and manifestation of congenital abnormality. Historically birth defects have always attracted the attention of lay persons as well as the scientific community. People have attributed birth defects to divine retribution, to bad food and maternal alcohol consumption during pregnancy. Once Mendel proposed his theory of inheritance, every instance of birth defect was attributed to a genetic defect in either parent. Until 1940, it was generally believed that fetal membranes could provide protection against teratogenic agents. This belief was changed with time when these agents were put to serious scrutiny. In 1941, Gregg first documented evidence to show that rubella virus could induce developmental defects of the fetal eyes, heart and ears (Gregg, 1941). Lenz (1962) and McBride (1961) observed that maternal consumption of thalidomide during pregnancy induced malformations of the limbs in human fetuses. About 7 to 9% of all human cogenital malformations are now known to be caused by drugs, viruses and other environmental agents (Persaud, 1990 and Thompson et al., 1991). Smith and his associates found an association between gestational hyperthermia and myelomeningocele, severe mental deficiency, microphthalmia, midface hypoplasia, distal limb defects and anomalies of the central nervous system in the offspring (Chance and Smith, 1978; Smith et al., 1978; Pleet et al., 1981). These finding were similar to those found in pigs and rats (Edwards et al., 1995). Cogential malformations could be of a single or multiple and minor or major types.

The causes of birth defects are divided into three groups: (i) genetic, (ii) environmental and (iii) multifactorial inheritance factors. For most of the congenital malformations, the exact causes are not known. They are possibly the result of a complex interaction between genetic and environmental factors. That is, they are of multifactorial inheritance. Environmental factors, known as teratogenic agents (because of their ability to induce cogenital malformations) act at critical periods during development. (Moore and Persaud, 1998). The results of such exposure on fetal development depend on the chemical or physical nature of the teratogen, the dose, and the genetic make up of the embryo, the genetic constitution of the mother and the developmental stage at which exposure occurs. The biochemical nature of the agent determines the rate of absorption of the agent and its

rate of transport from maternal into fetal compartment. Teratogens differ in their ability to initiate fetal responses. Some teratogens induce cell death, some inhibit cell proliferation and yet others interfere with cell differentiation, migration and developmental interactions between tissues. Often teratogens elicit multiple responses of embroyonic tissues. The teratogenic outcome manifests in fetal abnormalities, intrauterine growth retardation and functional disorders of the offspring. Teratogenic outcome is also reported to be species-specific and within a particular species strain differences are known to exist. Teratogenic exposure at preimplantation period of development generally results in either embryonic death or produce no effects at all. This is known as the "all or none" response. On the other hand, exposure to teratogenic agents during organogenesis often results in fetal malformations (Wilson, 1973).

Of the numerous environmental agents some are proven to be teratogenic to human embryos; others are suspected to be harmful. There are numerous physical and chemical agents whose effects on human development are unknown. Maternal hypothermia has been implicated in human and experimental teratogenesis (see below).

1. Heat Stress

Normal body temperature is maintained by physiological homeostatic mechanisms unless an environmental condition alters it. It is important to mention here that normal body temperature exhibits diurnal cycles; it rises in the morning to reach its maximum levels in the afternoon and then falls in the evening (Terai et al., 1985). Hyperthermia is defined as an elevation of normal body temperature above normal range (Walsh et al., 1998). Heat shock response is defined as changes in the homoeostasis (Buckiova and Jelinek, 1995) that involve activation of heat shock protein (HSP) expressing genes (Santoro, 2000). Heat shock proteins are responsible for rapid adaptation to changed environment (Nishizawa et al., 1999; Welch and Suhan, 1986 and Frank et al., 1999). For example, the expression of HSP 70kD occurs in the brain cells only under heat stress (Lee et al., 1992; Quraishi and Brown, 1995). However, the HSP 90kD is expressed under normal conditions and its expression is enhanced by heat stress (Lee et al., 1992). Heat shock protein 70kD localizes at the synapse to provide protection against heat-induced damages (human) (Bechtold et al., 2000). Heat shock protein 70kD is also important in protein folding (Diehl and Schmidt, 1993) and protein undernourishment induces it in the rat (Kawai et al., 2000). However, HSP 90kD is bound to inactive form of the glucocorticoid receptor to provide

adaptive response against adverse conditions (rat) (Ali and Vederckis, 1990; Vamvakopulos, 1993).

Hyperthermia in laboratory animals induces physiological changes such as increases heart rate (Hale et al., 1957), cardiac output, oxygen demand, cutaneous blood flow (Shapiro and Seidman, 1990), ventilation (Gautier, 2000), respiratory alkalosis (rabbit) (Daghir, 1995), sweating (Sugimoto et al., 1996), dehydration (Cortes et al., 2000), enhanced water consumption, urine production (Belay and Teter, 1993), metabolic rate (Gautier, 2000), plasma osmolality, glucose, urea, lactate levels (Abdelatif and Modawi, 1994), increased plasma concentrations of adrenocorticotrophic hormone (Siegel et al., 1979), cortisol (corticosteron), aldosterone, rennin (Finbeg and Berlyne, 1977), growth hormone and thyroid stimulating hormone (Parkhit and Jonhson, 1969), hepatic enzymes (Abdelatif and Modawi, 1994) and decreases blood pressure (Whitto et al., 1964), the pH (Welch, 1992), food consumption (Arjona et al., 1990), ATP levels (Welch et al., 1992), and plasma concentrations of sodium, magnesium, total calcium and inorganic phosphorous (Arjona et al., 1990). Furthermore, the physical activity of the animals is also reduced due to release of β-endorphin from the pituitary under heat stress (Galina et al., 1982). On the other hand, hyperthermia in humans increases blood pH and decreases blood CO_2 tension, peripheral vasodilation and diastolic blood pressure (Smith et al., 1978).

Hypertheremia can be induced in animals either by immersing in hot saline (Editorial, 1978), bacteriological incubator (Kilham and Ferm, 1976), warm air chamber (Kimmel et al., 1993a), water bath (Germano et al., 1996) or hot environment (Bell et al., 1989). Heat stress also can be caused in human by fever (Shaw et al., 1998), use of electric blanket (Milunsky et al., 1992), sauna (Tikkanen and Heinonen, 1991) or hot tub (Milunsky et al., 1992) (Table 1).

2. Teratogen Response to Heat Stress

Heat stress has been shown to be a potent teratogen in humans (Edwards, 1986; Edwards et al., 1995) and in experimental animals such as mice (Webster and Edwards, 1984), rats in *vivo* (Edwards, 1967; Webster et al., 1985; Germain et al., 1985) and in *vitro* (Walsh et al., 1985; Kimmel et al., 1993a), guinea pigs (Upfold et al., 1989; Breen et al., 1999), hamsters (Kilham and Ferm, 1976), monkeys (Hendrickx et al., 1979) and in incubating chicken (Edwards et al., 1995).

Following maternal heat exposure, craniofacial defects such as cleft palate, cleft lip, microcephaly, excencephaly, encephalocele, anencephaly, hydranencephaly, menigocele, orofacial clefts, microphalmia and maxillary hypoplasia have been observed in rat (Germain et al., 1985; Webster et al., 1985), mouse (Shiota, 1988), guinea pig (Smith et al., 1992), hamster (Kilham and Ferm, 1976) and human (Milunsky et al., 1992) fetuses. Limb defects have been observed in rats (Breen et al., 1999; Cuff et al., 1993), chick (Nilsen, 1969), human (Martinez-Frias et al., 2001) and guinea pig (Edwards et al., 1984) with temperature ranging from 38.9 to 42.9°C. However, axial skeletal malformations were specific to the rat (Kimmel et al., 1993b) and mouse (Li et al., 1997) when body temperature was increased by 2.5°C above the normal. Guinea pig is the only species that has been reported to show kyphosis and scoliosis under maternal heat stress during gestation (Smith et al., 1992). Cardiovascular defects were seen in chick (42°C) (Nilsen, 1984), human (about 38.9) (Tikkanen and Heinonen, 1991) and guinea pig embryos (about 3°C above normal temperature) (Smith et al., 1992). Rat (Mirkes, 1985; Walsh et al., 1987), sheep (Bell et al., 1989) and mouse (Shiota and Kayamura, 1989) fetuses were observed to be growth retarded when maternal temperature reached 40°C or higher. Mouse and human offspring show learning disorders due to prenatal maternal heat stress (Smith et al., 1978; Shiota and Kayamura, 1989). Heat stress causes cell death and disturbance in cell proliferation, neuronal migration neuroepithelial necrosis, and inhibition of development in hamsters (Kilham and Ferm, 1976), rats (Breen et al., 1999; Kimmel et al., 1993a) and mouse embryos (Shiota, 1988). Only heat stressed guinea pig fetuses were found to have hypoplastic teeth (Edwards, 1972) and branchial arch defects (Smith et al., 1992). Furthermore, eye defects were observed in heat stressed chick (Nilsen, 1969) and rat (Webster, 1985). However, only hyperthermic chick embryos were observed to have spinal cord defects (Nilsen, 1969; Buckiova and Jelinek, 1995).

Elevation of temperature stress at critical stages of embryonic development produces specific developmental defects that cannot be produced at other stages. In general, embryogenesis starts from head and progresses to the caudal region and according to the developmental stage at which heat exposure occurs fetal malformations are found to vary. That is to say that hyperthermia produces stage-specific malformations.

Craniofacial defects have been observed following maternal hyperthermia on gestation day (GD) 9 in rat (Germain et al., 1985; Webster et al., 1985), GD 8.5 in mice (Shiota, 1988), on GD 11-14 in guinea pig (Smith et al., 1992), on GD 8 in hamster

(Kilham and Ferm, 1976) and during the first trimester of human (Milunsky et al., 1992) pregnancy. In addition, limb defects have been observed in embryos heated on GD 9-11 in the rat (Breen et al., 1999; Cuff et al., 1993), 1-6 days of incubation in chick (Nilsen, 1969), GD 20-23 in guinea pig (Edwards et al., 1984) and 1-4 month in human pregnancy (Martinez-Frias et al., 2001) with temperature ranging from 38.9 to 42.9°C. However, axial skeletal malformations were observed when exposed to maternal heat stress on GD 10 rat (Kimmel et al., 1993b) and GD 8.5 mouse (Li et al., 1997) when body temperature rose to 2.5°C above normal temperature. Guinea pig fetuses showed kyphosis and scoliosis under heat stress condition on GD 11-14 (Smith et al., 1992). Cardiovascular defects were seen in chick when heat stressed on 3rd day of incubation (Nilsen, 1984), GD 11-14 in guinea pig (Smith et al., 1992) and early development embryos in human (Tikkanen and Heinonen, 1991). Fetuses of rats on GD 9.5 (Mirkes, 1985; Walsh et al., 1987), sheep between GD 64 to 141 (Bell et al., 1989) and mice GD 12-15 (Shiota and Kayamura, 1989) that have been heat stressed at 40°C or higher were observed to be growth retarded. On the other hand, offspring of mouse heat stresses on GD 12-15 and week 4-6 in human show learning disorders (Smith et al., 1978; Shiota and Kayamura, 1989). Maternal heat stress applied on GD 8 in hamsters (Kilham and Ferm, 1976), on GD 10 in rats (Breen et al., 1999; Kimmel et al., 1993a) and on GD 8.5 in mice (Shiota, 1988) resulted in defects of cell growth and proliferation. Eye defects were observed after heat exposure on day 1-6 incubation in the chick (Nilsen, 1969) and on GD 9-10 in the rat embryos (Webster et al., 1985). However, spinal cord defect was only observed in chick embryos when heat stress was applied on day 1-6 of incubation (Nilsen, 1969; Buckiova and Jelinek, 1995).

2. A. Genotype

Genotype play an important role in determining the sensitivity of the critical period for the induction of specific malformations as it has been shown in animal studies (Germain et al., 1985). Therefore, the types and severity of malformations depend on the animal genotype. Furthermore, depending on the genotype the normal body temperature varies whereas it is 38.5°C in rats, 39°C in guinea pig (Germain et al., 1985) and approximately 38.3°C for mice (Shiota, 1988). Although there are general similarities in heat-induced malformations in all species, each species has its own characteristics (Edwards et al., 1995) and developing brain was the most susceptible to heat. For example,

in all mouse strains neural tube defects, mainly excencephaly were the major teratogenic effects due to heat stress (Webster et al., 1984).

2. B. Threshold dose of hyperthermia

The threshold dose of hyperthermia is defined as the lowest dose required to produce a given defect in a significant number of exposed embryos. There is no single or simple means of quantifying the dose, which is a function of elevation and duration of temperature (Graham and Edwards, 1998). Edwards et al., (1995) defined the thermal dose as the amount of heat delivered to an embryo and is a product of the abnormal elevation of temperature with the duration of elevation. The parameters that determine the teratoenicity of hyperthermia include temperature elevation, duration of exposure (Rao et al., 1990) and the number of exposures (Edwards, 1981; Shiota, 1988).

3. Influence of Heat stress on Maternal Hormonal Profile

Many studies reveal the effects of heat stress on maternal body temperature, food and water consumption, body weight gain and other signs of toxicity but not changes in hormonal profile such changes might impact on fetal growth and skeletal development therefore it is important to look at the hormonal profile of heat stressed pregnant animals.

Endocrine system is involved in all aspects of pregnancy, including implantation, placentation, maternal adaptation, embryonic development and fetal growth and differentiations (Griffin and Ojeda, 1992). Hormones often work in antagonistic, agonistic or synergistic ways to precisely control the processes of rapid adaptation of the organism to such changes in the environment. Regulation of bone development involves hormonal sensitivity of chondrocyte cells during the process of cell differentiation, replication, maturation and matrix calcification. Hormones involved in bone developmental processes include thyroxin (stimulate differentiation and growth of cartilage), 1, 25-hydroxyvitamin D_3 (facilitates maturation and calcification of cartilage), glucocorticoids, androgens (influence cessation of growth and remodeling), somatomedins and parathyroid hormone-calcitonin system (Genser, 1986). Genser (1986) stated that maternal circulating hormone levels during pregnancy and fetal skeletal development maybe related to each other and could be used as a good indicator of intrauterine development.

3. 1. T_3 and T_4

The hypothalmo-pituitary-thyroid axis is an important element in body temperature regulation that contributes to maintaining basal metabolic rate (Oppenheimer, 1979). Thyroid hormones (T_3 and T_4) play an important role in adaptation, to altered environmental temperature (Bobek et al., 1996) by either reducing or stimulating heat tolerance (Bowen et al., 1985). It is important to mention here that heat stress causes significant decreases in serum thyroxin (T_4), triiodothyronine (T_3) (Horowitz and Meiri, 1985), thyroid stimulating hormone (TSH) in rats (Tal and Sluman, 1975) and plasma triiodothyronine (T_3) in cows (Baccari et al., 1983), chickens (Arjona et al., 1990; Yahav and Plavnik, 1999) and ewes (Bell et al., 1989). However, cold stress leads to significant increases in serum thyroxin and triiodothyronine levels (Khalil, 2002).

There is a strong relationship between fetal and maternal circulating thyroid hormones before fetal thyroid gland begins to function. Placenta controls the gradual development of the fetal hypothalamic-pituitary-thyroid axis by influencing the transfer of thyroxin from the mother to the fetus during gestation (Vulsman and Kok, 1996). Maternal thyroid hormones can cross the placenta, therefore, maternal thyroid hormonal deficiency leads to deficiency of thyroid hormones in fetal tissue that leads to developmental delay (Baccari et al., 1983; Escobar et al., 1985), disturbance of brain development (Sampson et al., 2000) and reduction in placental growth (Bell et al., 1989). Changes in fetal thyroid structure such as accumulation of colloid and flattened of secretary epithelial cells have also been reported (Andrianakis et al., 1990; Khalil, 2002). Maternal thyroid hormones during early pregnancy are essential for the development of early fetal brain until the fetus is completely self-supporting (Vulsman and Kok, 1996; Pop et al., 1999). Pop et al., (1999) found that impairment the production of maternal thyroid hormones was associated with severely impaired neurological development of the offspring. Furthermore, the early disturbances in neuronal differentiations were not corrected by the onset of fetal thyroid hormone secretion in rats (Sampson et al., 2000). The hypothyroidism is associated with abnormalities in structure and function of the skeletal muscles in rats (Janssen et al., 1978) and in humans (Khaleeli et al., 1983; Lomax and Robertson, 1992).

Thyroid hormones play an important role in bone formation or resorption by acting in a direct and an indirect way on bone cells *in vitro* (Allain et al., 1992; Conaway et al., 1998) or in *Vivo* (Mundy et al., 1976). Thyroid hormones may act on bone cells either indirectly by increasing secretion of growth hormone (GH) and in turn insulin-like growth factor-1 (IGF-1) (Khalil, 2002), or directly by influencing target genes via specific nuclear

receptors which are still not yet understood (Abu et al., 1997). Hyperthyroidism is characterized by increased bone turnover and resorptive activity (Langdahl et al., 1997). The effect on bone resorption of the thyroid hormones is dependent on increased cellular replication, perhaps of osteoclast precursors, or other bone cells involved in the resorptive process (Conaway et al., 1998). Causes of hypothyroidism status include iodine deficiency (Hollowell and Hannon, 1997; Glinoer and Delange, 2000), excess of thyroid binding globulins (Khalil, 2002) or impaired thyroid response to thyroid stimulating hormone (Tonacchera et al., 2000).

3. 2. Glucocorticoides

Corticosteroids are steroid hormones produced by the cortex of adrenal glands and include a number of hormones (glucocorticoides) important on the metabolism of glucose and other organic nutrients (Vander et al., 1994). They promote hydrolysis of muscle proteins to amino acids and in turn increase the level of glucose when these amino acids are converted to glucose in the liver. Because of wide distribution of the glucocorticoid receptors they are able to impact on bone and calcium metabolism (Reid and Fracp, 2000). Furthermore, heat stress and other forms of stress increase glucocorticoid activity in cells (Li et al., 2001). In *vitro* and in *vivo* studies have revealed the direct actions of glucocorticoids on the osteoblast cells, in decreasing type I collagen and increasing collagenase. Additionally, the indirect actions of glucocorticoids are mediated by reducing activities of growth factors (IGF I and IGF II) and causing reduction in collagen synthesis (Canalis, 1996). Glucocorticoids also interact with bone metabolism by reducing osteoblast number and bone matrix synthesis (Reid and Fracp, 2000). Canalis (1996) reported that the continuous exposure of skeletal tissue to excess amount of cortisol or corticosterone resulted in osteoporosis. Interleukin-6 (IL-6) is produced by osteoblasts, macrophage, lymphocytes, monocytes and it induces bone resorption (Ishimi et al., 1990, Kalil, 2002). The stimulation of bone resorption activity is related to the increase of osteoclast activity. The induction of interleukin-6 (IL-6) receptors in the skeletal cells play a central role in bone resorption, whereas IL-6 known to induce osteoclast employment (Jilka et al., 1992; Geisterfer et al., 1995; Dovio et al., 2001 a, b). Heat stress produces higher serum concentrations of interleukin-6 (Chung et al., 1999). The excess level of cortisol has an inhibitory effect on IL-6 production from osteoblast in vitro (Dovio et al., 2001). High levels of glucocorticoids are believed to alter bone remodeling by decreasing bone formation and increasing bone resorption (Swolin-Eide and Ohlsson, 1998)

Devarajan and Benz (2000) found that glucocorticoids decreased renal tubular calcium reabsorption and led to hypercalciuria. More chronically they interfere with intestinal calcium absorption, alter vitamin D metabolism and lead to secondary hyperparathyroidism (Griffin and Ojeda, 1992). Circulating cortisol is a very sensitive index of heat stress and an elevated level of cortisol is seen at the onset of excessive heat stress (Follenius et al., 1982). In addition, significant increases in plasma cortisol level and urinary excretion of water, sodium, and calcium but not potassium are found after heat stress. Urinary calcium/magnesium ratio is also significantly elevated (Marya et al., 1987).

3. 3. Parathyroid hormone and Osteocalcin

3. 3. A. Parathyroid hormone

The parathyroid glands are small bodies near the thyroid gland. They secrete parathyroid hormone (PTH) (Khalil, 2002). Parathyroid hormone plays an important role in the maintenance of a stable internal environment by sensing changes in concentration of calcium (Ca^{++}) ions (Brown et al., 1993). Furthermore, within minutes of a decrease in calcium, PTH promotes an increase in reabsorption of calcium from distal renal tubules and release of calcium from the bone (Gundberg et al., 1991). Serum calcium level has a diurnal rhythms that involve transfer of Ca^{++} into the bone, releases of Ca^{++} from the bone and then bone resorbing activity (Shinodo and Stern, 1992). Absorption and excretion of calcium increases during pregnancy whereas bone turnover increases during late pregnancy in human (Cross et al., 1995). Abnormal regulation of PTH secretion by Ca^{++} plays an important role in the pathophysiology of hypercalcemia (Cetani et al., 2000). Calcium–sensing receptor interacts with extracelluar calcium and is expressed in parathyroid cells and C-cells (Brown, 1999). Down-regulation of calcium sensing receptor plays an important role in the abnormal secretary and growth patterns of parathyroid gland (Gogusev et al., 1997).

Some studies suggest that there is no relationship between serum PTH and serum total calcium (Brent et al., 1988). A recent study has shown that PTH applies its regulatory effects on calcium homeostasis by stimulating the release of calcium from the skeleton. Parathyroid hormone stimulates bone resorbtion indirectly by inducing the production of osteoblastic cell, which recruit and activate the bone-resorbing cell, the osteoclast. More recently, it has been demonstrated that osteoblast cells in response to PTH produce the interleukin-6 (IL-6), which potently induces osteoclastogenesis. Thus IL-6 may play a permissive role in PTH induced bone resorption (Grey et al., 1999).

3. 3. B. Osteocalcin

Osteocalcin (OC) or non-collagenous protein (NCP) is a bone-specific extracellular matrix protein and it is synthesized only by osteoblasts (Ducy et al., 1996; Brown et al., 1984). It constitutes 25% of the non-collagenous matrix protein of bone (Brown et al., 1984; Delmas et al., 1983). Douglas et al., (1996) reported that serum osteocalcin values were significantly greater in spring than in autumn. This bone-specific protein concentration in blood is a direct reflection of osteoblastic activity and bone formation (Gundberg et al., 1991). It also serves to promote an initial bone response to physiological stress before the normal hormonal regulations are elicited (Gundberg et al., 1991). Osteocalcin normaly functions to limit bone formation without impairing bone resorption or mineralization (Ducy et al., 1996). Cole et al., (1987) suggested that OC measurement was useful in investigations of bone mineral metabolism during pregnancy. Plasma osteocalcin levels increased steadily with fetal age during prenatal period (Verhaeghe et al., 1990).

It is important to mention that OC serum concentration is capable of predicting remodeling rates in postmenopausal osteoporosis (Brown et al., 1984). Serum levels of OC are used to determine whether age-related bone loss results from increased bone resorption, decrease in bone formation or both (Delmas et al., 1983). High serum osteocalcin levels are an index of low skeletal mass. Yasumura et al., (1987) found that there was a relationship between total body Ca^{++} and osteocalcin. In general, the osteoporotic women had low total body Ca^{++} values and high ostocalcin levels.

Maternal circulating 1, 25 (OH) 2D (vitamin D) is an important determinant of fetal plasma 1, 25 (OH) 2D (vitamin D) in the rat, since both are correlated (Verhaeghe et al., 1988). Specific vitamin D cytosol binding sites were found in skeleton (ribs and vertebral bodies) (Nguyen et al., 1987). Although serum levels of osteocalcin were thought to be an indicator of osteoblastic activity and bone formation, there was little information concerning the acute effects of changes in calcium or PTH level on circulating concentration of osteocalcin (Gundberg et al., 1991). However, Cole et al., (1987) demonstrated that no significant correlations were found between maternal osteocalcin concentrations and serum phosphorus, alkaline phosphatase (bone biochemical marker), or parathyroid hormone, but significant negative correlations were found between osteocalcin and total calcium or total protein in human. Clinical hyperparathyrodism is always associated with increased serum osteocalcin levels (Price et al., 1980). Patricia et al., (1988) demonstrated that administration of physiological to low pharmacological dose of

corticosterone elicited a time and dose dependent decrease in serum ostocalcin in vivo. In addition, the response to cold exposure also decreased osteocalcin levels. These changes observed in response to an extremely well defined hormonal system imply an important role for corticosteroids in the control of serum osteocalcin level. Bodine et al., (1996) concluded that vitamin D increased osteocalcin secretion in culture when the cell line is maintained at 40°C. Furthermore, the cells expressed very low basal levels of alkaline phosphatase activity in addition to high amount of osteocalcin and enhancement of parathyroid hormone in response to vitamin D_3. Higher osteocalcin levels in umbilical venous blood than in umbilical arterial blood suggested that the placenta may be the main source of osteocalcin in late fetal life (Seki et al., 1993). Uteroplacental blood flow in infants may result in reduced fetal-placental production of 1, 25-dihydroxyvitamin D, which results in low bone mineral content and low serum osteocalcin values; and fetal serum parathyroid hormone values may be relatively elevated because of reduced placental mineral supply (Namgung et al., 1993).

Table 1. Studies on hyperthermia induced cogenital malformation in experimental animals and humans.

Species	Developmental stage	Method of heat exposure	Temperature	Malformations	References
Rat	GD 9-14	Incubator	42.9°C for 40-60min.	Microphthalmia, anencephaly, tails defects, limb, toes, palate and body wall anomalies.	Edwards, 1968.
Rat	GD 9-10	Incubator	42°C for 15-20 min or at 43.5°C for 15min.	Decrease in head length, disturbance in number and development of somites, cell death, delayed in development of caudal neural tube, microphthalmia, head defect, encephalocele, maxillary hypoplasia, small eye, branchial bars defect and growth retardation.	Webster et al., 1985; Walsh et al., 1987; Mirkes, 1985; Cuff et al., 1993; Breen et al., 1999.
Rat	GD 9-10	Water bath	42°C for 2 or 15 min or 43°C for 7.5-8.0 min.	Encephalocele, facial cleft, maxillary hypoplasia, microphalmia, microphthalmia, microcephaly, gross reduction of the forebrain and open neural tubes, neural migration disorders, increase mortality and cell death (apiptosis).	Germian et al., 1985; Walsh et al., 1987; Rao et al., 1990; Germano et al., 1996; Mirkes et al., 1997.
Rat	GD 10	Brown-Fabro scoring system (*Vitro*)	42°C or 43°C to 10-25 min	Inhibition of development	Kimmel et al., 1993a.
Rat	GD 10	Incubator, warm air chamber	42°C for 5-20 min	Axial skeletal malformation and limb defect.	Cuff et al., 1993; Kimmel et al., 1993b; Breen et al., 1999.
Rat	GD 9.5	Exposed to heat then cultured for 12-48hr.	43°C for13 min.	Rostral neural tube defect.	Buckiova and Brown, 1999.
Mouse	GD 8.5	Water bath	42°C for 12.5-15min or 43°C for 7.5-10min.	Vertebral transformation, disturbances in cell proliferation, embryonic neuroepithelial necrosis, excencephaly, anencephaly, encephalocele and facial cleft.	Li et al., 1997; Shiota, 1988.

Table 1. Continued.

Mouse	GD 12-15	Water bath.	42°C or 43°C for 10 min.	Growth retardation, reduction in brain size and reduction in learning capacity.	Shiota and Kayamura, 1989.
Rat, guinea pig	GD 9-10	Incubator	42°C for 1hr daily.	Anophthalmia, anencephaly, microencephaly, hydranencephaly, hypoplasia of teeth and growth retardation.	Edwards, 1972.
Guinea pig	GD 11-14	Incubator	3.4-4.0°C above normal temperature.	Anterior neural tube defect, kyphosis-scoliosis, branchial arch defect and pericardial edema.	Smith et al., 1992.
Guinea pig	GD 20-23	Incubator	42.6°C -42.9°C for 1h.	Clubfoot, less brain weight, exomphalos and hypodactyly.	Edwards et al., 1984.
Hamster	GD 8	Incubator	41°C for 1.0-1.25hrs.	Disturbance of cell proliferation, microcephaly, excenphaly and encephalocele.	Kilham and Ferm, 1976.
Sheep	Between day 136-141 of pregnancy	Hot environment	40°C for 9h/d	Fetal growth retardation.	Bell et al., 1989.
Chick	GD 1-6	Egg Incubator	39°C to 43°C for 15min-2 days or 3 days.	Head, eye, spinal cord and limb and aortic arches defects.	Nilsen, 1969; Nilsen, 1984; Buckiova et al., 1995.
Human	1-3 month.	Electric blanket.	≥ 38°C	Neural tube defect and orofacial cleft.	Milunsky et al., 1992; Shaw et al., 1999.
Human	1-3 month.	Fever	≥ 38.9°C for 2 day	Cleft lip and cleft palate, anencephaly, mental deficiency, microphthalmia, midface hypoplasia, limb deficiencies and transformations of axial skeleton.	Smith et al., 1978; Layde et al., 1980; Milunsky et al., 1992; Lian et al., 1997; Chambers et al., 1998; Shaw et al., 1998; Martinez-Frias et al., 2001.
Human	1-3 month.	Sauna	38.9-43°C for 15min	Anterior neural tube defect (Anencephaly), Posterior encephalocele, menigocele and cardiovascular malformation.	Halperin and Wilroy et al., 1978; Miller et al., 1978; Tikkanen and Heinonen, 1991; Milunsky et al., 1992.

OBJECTIVES

The main objective of the present study is to determine the effects of maternal heat stress during pregnancy on fetal axial skeletal development and to explore some of the possible maternal and placental responses that can explain these effects. Thus additional objectives included evaluation of the possible alterations in maternal hormonal profile due to heat stress and effects of heat stress on different zones of placental and their relation with fetal malformation.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Materials

The Department of Biology, Faculty of Science and Department of Anatomy Faculty of Medicine and Health Sciences provided standard laboratory chemicals and equipment for this study. Enzyme link immunosorbant assay (ELISA) kits for thyroxin and triiodothyronine were purchased from dbc-Diagnostics Biochemical Canada Inc. (London, Canada). Osteocalcin EIA (Enzyme immuno-assay) kit was obtained from Biomedical Technologies Inc. (Massachusses, USA). Glucose commercial determination kit (Lyon, France), and calcium determination kit were acquired from bioMérieux (Lyon, France).

II. Animals

The Wistar rats used in this study were originally bought from Harlan Olac (England) and raised in our local animal house facility. Animals were housed in solid–bottom polypropylene cages containing heat-treated wood chips under conditions of constant temperature (25±1°C) and humidity (> 60%) and maintained on a 12-hour light dark cycle. The standard laboratory chow and tap water were provided *ad libitum*. Virgin female (200-250 gm) were mated with males overnight. The following morning vaginal smears were examined. The day on which sperm were found in the vaginal smear was considered day 0 of gestation. Pregnant animals were kept in groups of three per cage.

III. Heat–stress Procedure

A total of 74 animals were randomly assigned to three groups: (a) non-stressed (control, n=20), (b) heat stressed group I (41°C, n = 44) and (c) heat stressed group II (42°C, n = 10). The heat exposure lasted an hour starting 9.30 a.m on day 9 of gestation.

Experiment I

The effect of heat-stress on maternal physiological parameters was carried out using 40 pregnant rats. Animals were randomly assigned to two treatment groups, control (non-stressed, n = 10) at 25°C and heat-stressed (41°C, n = 30) groups. Animals were placed in a polypropylene cage containing heat-treated bedding chips in an incubator at the forementioned temperature. The temperature of the incubator was monitored continuously.

Rectal temperature was measured before and after heat stress by using Phillip's thermometer probe. An increase in body temperature of about 2 degrees was considered stress on experimental animals.

Experiments II

The effect of heat-stress on fetal abnormalities was conducted using the previously mentioned conditions. However, heat-stressed animals were exposed to 41°C (n = 14) and 42°C (n = 10) to demonstrate the extreme changes and the severity of skeletal malformations. Maternal animals were observed on gestation day 10 and 11 following heat exposure for clinical signs of heat stress such as exhaustion, recovery or death, differences in rectal temperature, food and water intake and changes in body weight.

IV. Maternal Physiological Parameters

Blood sample collection

Immediately after an hour of heat stress or sham treatment, blood samples were collected from the orbital veins in plain vials under ether anesthesia. The blood samples were collected from thirty heat stressed (41°C) animals and ten control animals. The blood was allowed to clot in centrifuge tubes and then centrifuged at 3000 r.p.m for 10 minutes at room temperature. Fresh serum samples were used for the determination of glucose and calcium. The rest of the serum was kept in a deep freezer at –85 °C until assayed for thyroxin, triiodothyronine and osteocalcin.

Glucose Assay

The glucose concentration was determined by glucose oxidase method (Trinder, 1969) by using a commercial kit (bioMérieux, Lyon, France). Standard curve was prepared as described by manufacturer. 10µl of serum was mixed with the reagent and incubated at 37°C for 10min. Thereafter, glucose concentration was measured as a function of the release of H_2O_2 using spectrophotometer (Photometer 4010) at 505 nm against reagent blank. The reagent buffer consisted of phosphate buffer pH 6.5 (225 mmol/l), amino-4-antipyrine (0.3 mmol/l), phenol (8.5mmol/l), EDTA (5 mmol/l), peroxidase (≥ 300 U/l) and glucose oxidase (≥ 100%).

Calcium Assay

The serum calcium concentration was determined by colorimetric method (Elveback, 1970). Serum calcium was estimated by using Ca^{++} determination kit (bioMérieux, Messorie, USA). Standard curve was prepared as described by the manufacturer. All glassware used in this assay were washed in 1N hydrochloric acid and rinsed in distilled water as well as disposable laboratory ware. Briefly, 50µl of serum was mixed with 2-5ml of methylthymol blue (80 mg/l) and 8-hydroxyquinolein (200 ml/l) and 2.5 monoethanolamine (200ml/l). The mixture was mixed for 1 min and the color intensity was measured at 612 nm using spectrophotometer (Shimadzo, model UV-160A) against reagent blank. Standard reagent used was 10mg/100ml.

Thyroxine (T_4) Assay

The serum thyroxin concentration was determined by enzyme immunoassay method (Robins, 1973). Thyroxin level was estimated using ELISA kits dbc-diagnostics biochemical Canada Inc (London, Canada). Standard curve was prepared as described by the manufacturer. Sera (25µl) of control and treated rats were pipettes into breakapart wells. Working T_4-Enzyme (200µl) was added to all wells. Wells were then mixed and incubated for 30 minutes at room temperature (25 °C). After incubation, wells were decanted, washed three times and blotted on absorbent paper towels. TMB substrate reagent of (150µl) were added to all wells and incubated for 30 minutes at room temperature. Reaction was terminated by addition of stopping solution. Absorbance at 450 nm was measured within 20 minutes after adding the stopping solution.

Triiodothyronine (T_3) Assay

The serum Triiodothyronine concentration was determined by enzyme immunoassay method (Robins, 1973). Triiodothyronine level was estimated using ELISA kits dbc-diagnostics biochemical Canada Inc (London, Canada). Standard curve was prepared as described by the manufacturer. Sera (25µl) of control and treated rats were pipettes into breakapart wells. 150µl of the working T3 enzyme conjugate solution were added into each well. Wells were then mixed and incubated at room temperature for 60 minutes on a slow-speed shaker. After incubation, wells were decanted, washed three times and blotted on absorbent paper towels. TMB substrate reagent (150µl) were added to all wells and incubated for 30 minutes on a slow shaker. Reaction was terminated by addition of

stopping solution. Absorbance at 450 nm was measured within 20 minutes after adding the stopping solution.

Osteocalcin Assay

The osteocalcin serum concentration was determined by enzyme immunoassay method (Gundberg and Weinstein, 1986). Osteocalcin EIA kit was purchased from biomedical technologies Inc. (Stoughton, USA). Standard curve was prepared as described by the manufacturer. Microtiter plate was removed from the resealable bag. Diluent buffer (25µL), standards (25µL), samples (25µL) and controls (25µL) were added to appropriate wells followed by 100µL osteocalcin antiserum. The entire procedure was completed in 15 minutes then the plate was gently swirled for about 1 minute. Thereafter, the plate was covered tightly and incubated at 37 °C for 2.5 hours wells were then aspirated completely and washed 3 times with 0.3ml phosphate buffered saline (PBS). At the end of incubation time, streptavidin-horseradish peroxidase reagent (100µL) was added to all wells and then incubated at room temperature for 30 minutes. At this stage sufficient amount of 1: 1 mixture of TMB and hydrogen peroxide was made and stored in the dark. After completing the incubation with streptavidin-horseradish peroxidase the plate was washed three times with PBS. Substrate (100µL) was added in each well, and then incubated at room temperature in the dark for 10 minutes. At the end of the incubation period, stop solution (100 µL) was added to each well and absorbance was measured immediately at 450 nm.

IV. Morphological and Skeletal Studies

Fetus collection and observations

Animals were killed by cervical dislocation on gestation day 20. The uterus was cut open and number of implantations and resprotions were counted. Fetuses were removed from the uterus, dried of amniotic fluid and dissected free of their membrane. They were then weighted, and fixed in 95% ethanol for subsequent examination of external malformations. Skeletons were stained with Alcian blue (cartilage) Alizarin red-S (bone) as described by Inouye (Inouye, 1976), and modified in our laboratory. The specimens were stained with 70% ethanol containing 0.015% alcian blue, 0.005% alizarin red-S and 5% acetic acid at 37°C for 2-3 days. Then the specimens were cleared through ascending concentrations of aqueous glycerin in 0.5% KOH and finally stored in 100% glycerin and examined with a stereomicroscope.

VI. Histological Studies

Hematoxylin and eosin staining of placenta

Immediately after collection, the placentas were fixed in Gender's fluid (Appendix 1). Tissues slices were dehydrated in ascending concentrations of ethyl alcohol (70%, 80%, 90%, 100%) by using auto processing machine. They were then cleared in xylene and infiltrated with paraffin wax (Appendix 2). Sections (7μm) were cut and mounted on glass slides. They were subsequently dewaxed, rehydrate in descending concentrations of ethyl alcohol and washed in distilled water for 5min. Sections were then stained (Bancroft and Stevens, 1977) with hematoxylin for 5min then dipped into acid ethanol followed by eosin staining for 30seconds. Stained slides were dehydrated, cleared, mounted, and dried for final microscopic examinations.

Best's Carmine stain of placenta

Best's Carmine staining (Gretchen, 1979) was performed to detect the presence or absence of glycogen granules in the placenta (Appendix 3). Briefly, paraffin sections were dewaxed, rehydrated, stained with hematoxylin (5min), washed with running water (5min), and placed in Best's carmine working solution (30min, Appendix 4). Slides were then treated with differential fluid (Appendix 5), dehydrated, cleared and mounted for microscopic examination.

VII. Electron Microscopic Studies

Placental tissues from rats that were exposed to 41°C, together with sham controls were processed and fixed in McDowell and Trump fixation with continuous mixing for 1 hour at room temperature and allowed to stand overnight at 4°C. Samples were rinsed in phosphate buffer (0.1M) and stored at 4°C for further processing. Samples were treated with buffered 1% osmium tetraoxide for 1 hour at room temperature. Tissues were then washed with distilled water and dehydrated in ascending concentration of ethanol. Samples were then placed in two changes of propylene oxide. Thereafter samples were blocked in Agae 100 resin. Ultrathin sections were cut using diamond knife and examined with Philips CM10 Electron microscope.

Statistical Analysis

Statistical analysis for hormone, glucose, osteocalcin, calcium data was carried out using SPSS (Norusis, 1998), however, all others statistics were done by EPI6 INFO. Public domain software for epidemiology and disease surveillance (EPI6 INFO, 1996).

CHAPTER III

RESULTS

RESULTS

I. Maternal Effects

Pregnant Wistar rats were exposed to heat stress at 41°C or 42°C for one hour on GD 9. The animals whose rectal temperature reached about 2°C or more above normal body temperature showed signs of toxicity such as exhaustion, decreased water and food consumption, sweating and diarrhea. They also appeared to be sleepy and less active after being hyperactive for a few minutes. After 24 hours, water and food consumption rate became normal. Data of the present study (Table 2) indicate that exposure to 41°C caused a severe heat stress leading to a significant increase ($P < 0.05$) in serum glucose (119.78 ± 5.90 mg/dl) as compared to the control animals (98.47 ± 3.45 mg/dl). In addition, the serum calcium, T_3 and T_4 levels were significantly decreased ($P < 0.05$) in comparison to the control animals. The control animals had an osteocalcin level of 0.730 ± 0.015 ng/ml. Heat stress caused a significant increase ($P < 0.05$) in serum osteocalcin.

II. Fetal Effects

The animals that were subjected to 42°C showed significant reductions in the number of implantations, number of live embryos, and fetal and placental weights in comparison to control animals. There was a significant increase in resorption rate, malformations and growth retardation (Table 3, Figs 1-2). Fetuses at -1SD are -2SD from the mean of the control weights were regarded as growth retarded. It was observed that about 50% of 42°C group were at -2SD whereas about 40% of 41°C group were at -2SD level.

1. Morphological Malformations

The tip of the upper jaw was a little anterior to that of the lower jaw in control fetuses. In maxillary hypoplasia of experimental fetuses the tip of the upper jaw was found to be behind the tip of the lower jaw (Figs 2B, C). A large number of the experimental fetuses were found to have their lower jaw hypoplastic (Table 4). In such fetuses with mandibular hypoplasia, the lower jaw was considerably behind the tip of the upper jaw. In control fetuses, the ear is located at or above the trans-oral line, which passes along the oral fissure. But when the ear is located below the trans-oral line, then it is regarded as low set (Figs 3A). It is important to mention that usually low set ears were also associated with small size. The incidence of low set microtia increased with increase in temperature.

The control mandible and maxilla bones were well ossified. The mandible contained a ramus, body and symphysis menti. The hypoplastic condition of the maxilla and mandible were possibly the reasons why protrusion of the tongue occurred (Fig 3B). These results show the correlation between mandiblular and maxillary hypoplasia and tongue protrusion. The heat stress caused an increase in the incidence of mandibular and maxillary hypoplasia compared to the controls. But in comparison between 41°C and 42 °C, the 41°C group showed a higher incidence of maxillary-mandibular hypoplasia and tongue protrusion. Fetuses with excencephaly had the brain protruding outside the skull due to the absence of the cranial vault (Figs 2B-D, 3B). The protruded brain tissue had degenerated. Bleeding from the exposed brain resulted in blood stained amniotic fluid. Excencephalic embryos were growth retarded. Polyhydramnios was common in excencephaly. Both experimental groups showed a high incidence of excencephaly (Table 4).

The eyes remain closed in normal rat fetuses and open only after birth. The lens of normal fetuses is transparent. Heat stressed fetuses showed a low incidence of exopthamia with cataract. Fetuses with facial clefts, especially those with bilateral oblique facial clefts had cleft of the upper lip (Fig 1C). Facial clefts were sometime found to be associated with excencephaus. Fetuses of the 41°C group had a higher incidence of facial clefts than those of the 42°C group. Furthermore, both experimental groups showed a low incidence of cleft palate. The hyperthermic groups had a higher incidence of short tail than the controls (Table 4). One of the 42°C group fetuses had a skin appendage attached to the dorsal aspect of the right hindlimb (Fig 1D).

2. Skeletal Malformations

The Skull Bones

The control rat fetuses had well ossified bones of the skull that included the mandible, premaxilla, maxilla, zygomatic, nasal, frontal, parietal, interparietal, supra-occipital, exoccipital, temporal, tympanic ring, hyoid, ethmoid, presphenoid, basisphenoid and basioccipital bones (Figs 4A, 5A, 6A, 7A). No instance of hypoplastic or absent bones was noticed. The control fetuses showed a normal size of fontanelles and suturel lines (Table 5; Fig 6A).

The comparison between 41°C and 42°C groups showed variable responses in terms of skeletal defects (Figs 4, 5, 6). The control fetuses had well-ossified mandibles. The remnant of the Meckel's cartilage was only found near the symphysis menti. In contrast, a large number of the experimental fetuses were found to have the entire Meckel's cartilage persisting (Fig 5). An increased incidence of hypoplasia of premaxilla, maxilla, parietal and temporal bones was observed in 41°C group than in 42°C group. However, fetuses of 42°C group showed a higher incidence of hypoplasia of basisphenoid and exococcipital bones than those of the 41°C group. Fetuses of 42°C group showed a higher percentage of absent bones such as maxilla, zygmotic, parietal, frontal, interparital, supra-occipital, exoccipital, temporal, tympanic, hyoid, ethmoid and presphenoid than the 41°C group.

In both experimental groups, the fontanelles and sutural lines were wider, which indicated poor ossification of the cranial vault bones. On the other hand, the 42°C group fetuses were more hypoplastic and had only traces of ossification of the cranial vault (Figs 6B-D).

The Vertebral Column

The rat fetal vertebral column normally has 26 presacral vertebral arches and bodies that are distributed as follows: 7 cervical arches-bodies, 13 thoracic arches-bodies, 6 lumber arches-bodies and variable number of sacral and coccygeal arches-bodies. In our study the control vertebral column appeared to have well ossified vertebra (Table 6; Figs 9A, 10A, 11A, 15A).

In the experimental fetuses, the vertebral arches and bodies showed decrease in number and poor ossification (Figs 9B, C; 10B; 11B; 12A, B). Fetuses of 42°C group showed a higher incidence of hypoplatic cervical vertebral bodies than those of 41°C group. Also the percentages of absent cervical bodies were higher in 42°C groups than those in 41°C group. In fetuses of both experimental groups, there were fewer thoracic arches and bodies compared with those of the control group. The higher the temperature higher was the reduction in number of lumbar, sacral and coccygeal arches and bodies in experimental groups. The 42°C group was found to have a higher percentage of agenesis of sacral arches and bodies compared to those of 41°C and control group.

The Sternebare and Ribs

Sternum consisted of 6 sternebare in the control fetuses (Fig 13A). They also had seven pairs of sternal ribs. Control ribs and sternebare appeared normally ossified without any reduction in normal count. No instance of fused or hypoplatic ribs was found in control fetuses. The higher the temperature during hyperthermia, the higher was the incidence of skeletal defects (Table 7, 8; Fig 13). Malformations of ribs appeared usually in the form of hypoplasia or fusion (Table 7; Figs 13B, C; 14A, B). Hypoplastic ribs were more frequent in fetuses exposed to 42°C than those exposed to 41°C. However, fused ribs were increased in 42°C group. On the other hand, sternebare appeared hypoplastic (Fig 13B), split (Fig 13C), absent (Fig 14A), or in misaligned (Fig 14B) forms. Hypoplastic and split sternebare were more commonly observed in 42°C than in 41°C group (Table 8). There were no instances of hypoplastic, split and absent sternebare found in control fetuses.

The hip bones

The control hip bones of GD 20 fetuses consisted of ilium, ischium and pubis. These bones were well ossified and remained united by cartilage (Table 9; Fig 11). There was a higher incidence of hypoplastic ilium, ishchium and pubis in 42°C group compared with 41°C.

The limb bones

Forelimb skeletons of the control fetuses consisted of normally developed scapula, humerus, ulna and radius, metacarpals and phalanges (Fig 16A). The hindlimb skeleton consisted of femur, tibia, fibula, 4-5 metatarsals and a variable number of phalanges (Table 9, 10; Fig 11). Fetuses of 42°C group were observed to have a higher percentage of hypoplastic femur and tibia than those of 41°C group (Table 9). The 42°C group had a higher incidence of decreased number of metacarpals and phalanges than those of the 41°C and control groups (Table 10; Fig 16B). They also showed high incidence of absence of metacarpals, metatarsals and phalanges compared with 41°C. Tarsal bones were absent in both control and experiment groups.

III. The placenta

The rate placenta consists of two portions: a fetal portion and a maternal portion (Ross et al., 1989). Histologicllay it comprises three distinct zones. From maternal to fetal side, they are (1) deciduas basalis, (2) spongy zone and (3) labyrinthine zone (Davis and Glasser, 1968; Padmanabhan and Al-Zuhair, 1988; Padmanabhan and Al-Zuhair, 1990). The decidua basalis is found to consist of fibroblast-like cells and parallel bundles of collagen fibers embedded in a ground substance (Fig. 17A, B). The spongy zone is composed mainly of three types of cells: basophils, giant cells and glycogen cells (Figs. 17C, D). Clusters of glycogen cells (Fig. 17B; Figs. 18A, B, C) are observed to be surrounded by a layer of flat trophoblast cells and distributed between others types of cells. These clusters show a characteristic spongy appearance in H&E preparations (Fig. 17B). On the other hand, the labyrinthine zone comprises finger-like structures that extend from the spongy zone up to the fetal surface (Fig 19).

Viewed with an electron microscope, each labyrinth consists of capillaries embedded in a core of fetal mesenchyme covered with a trichorial membrane. The latter consist of three layers of trophoblast cells. Layer I is thin, often presents pores and directly faces the blood in the maternal sinusoid. Layer II contains glycogen granules, lipid and some secretary granules. Layer III adjoins the capillary endothelium and shares with it a common basal lamina. They contain pinocytic vesicles and a few glycogen granules (Fig 24).

In this study, the placentas of experimental animals had a lighter weight compared with the control group. In the hyperthermic group of placentas, the decidua were thicker than that of control group. There were large areas of hyalinization (Fig. 20C) and random lymphocytic infiltration. However, the spongy zone had high proportion of giant cells (Fig. 20D). Multinucleated giant cells were more abundant than those with single nucleus and the basophils were extremely numerous. The glycogen cell clusters were obviously reduced or absent over a large proportion of the basal zone (Figs. 21, 22). The loss of glycogen cell clusters might have led to the formation of cysts observed in this experiment (Fig. 22). The 42°C group had more degenerated glycogen cells than those of 41°C group. The labyrinthine zone showed reduction in thickness with much shorter labyrinths than those of control group and some parts of it appeared hyalinized (Fig. 23A). The columnar arrangement of labyrinths was altered and they were found to be either avascular or poorly vascularised. It was also characterized by the presence of fetal mesenchyme and

preivascular fibrosis (Figs. 23B, D). Giants cell appeared to have proliferated (Fig. 23C). The finger-like projections of this zone observed in controls (Fig. 19A, B) were also found to be altered. On the other hand, perivascular fibrosis was prominent.

Electron microscopic examination of the placentas showed a series of degenerative changes in experimental group compared with control group placentas. These changes included the presence of lymphocytic and phagocytic infiltrations, and accumulation of fat droplet in fetal mesenchymal cells. Fetal preivascular mesenchyme consisted of numerous of collagen in their matrix. In heat stressed group placentas, the trophoblast of the labyrinths had plenty of glycogen accumulations.

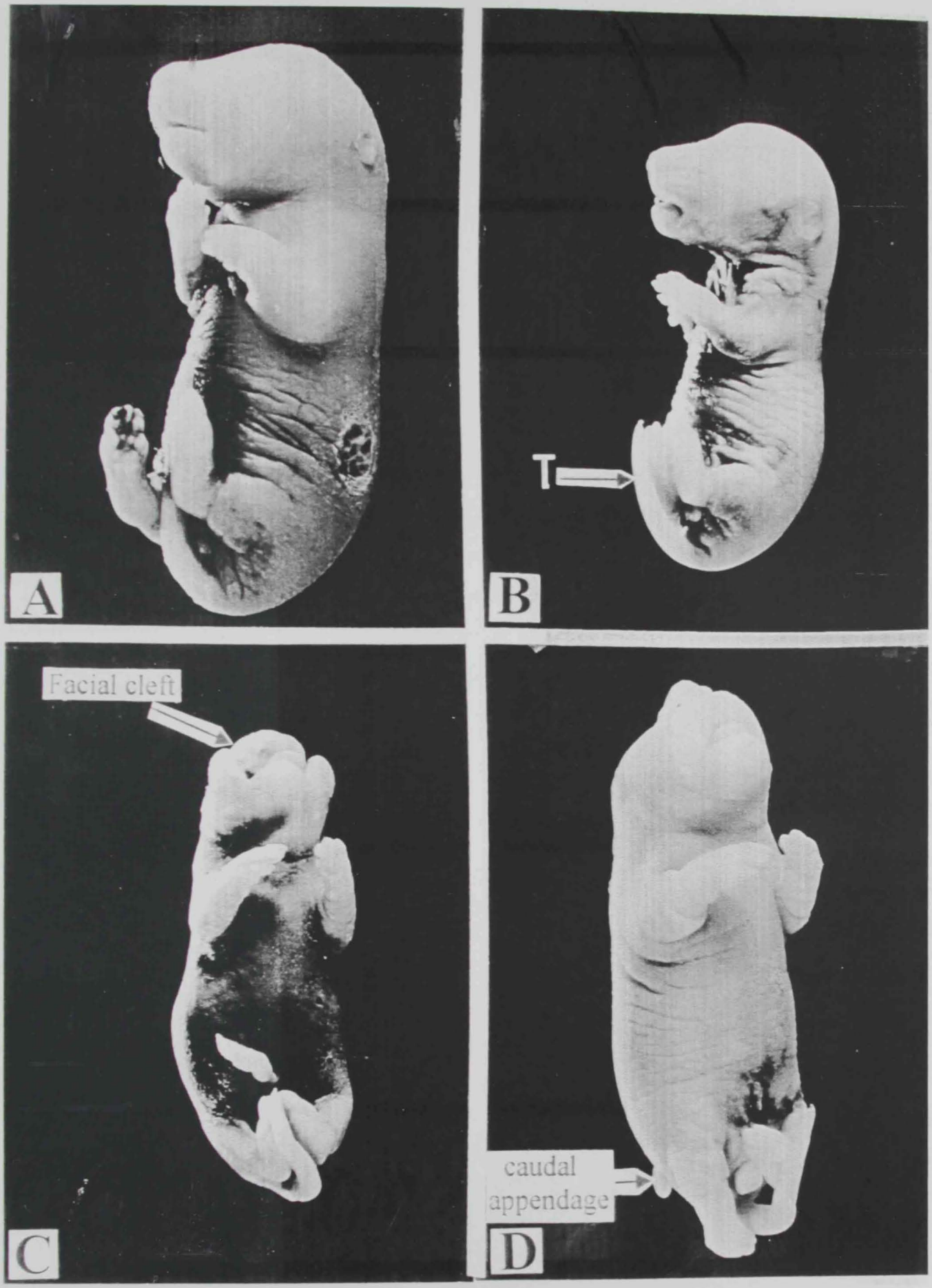

A
T
B
Facial cleft
C
caudal
appendage
D

Fig 1 Rat fetuses of gestation day (GD) 20 A Control B and D 42°C/1hr on GD 9, C 41°C/1hr on GD 9 Observe the short tail (arrow in B), growth retardation (B, C), oblique facial clefts involving the upper lips (C) and encephalocele (D) in heat stressed fetuses Fetus D presents a caudal appendage (arrow in D).

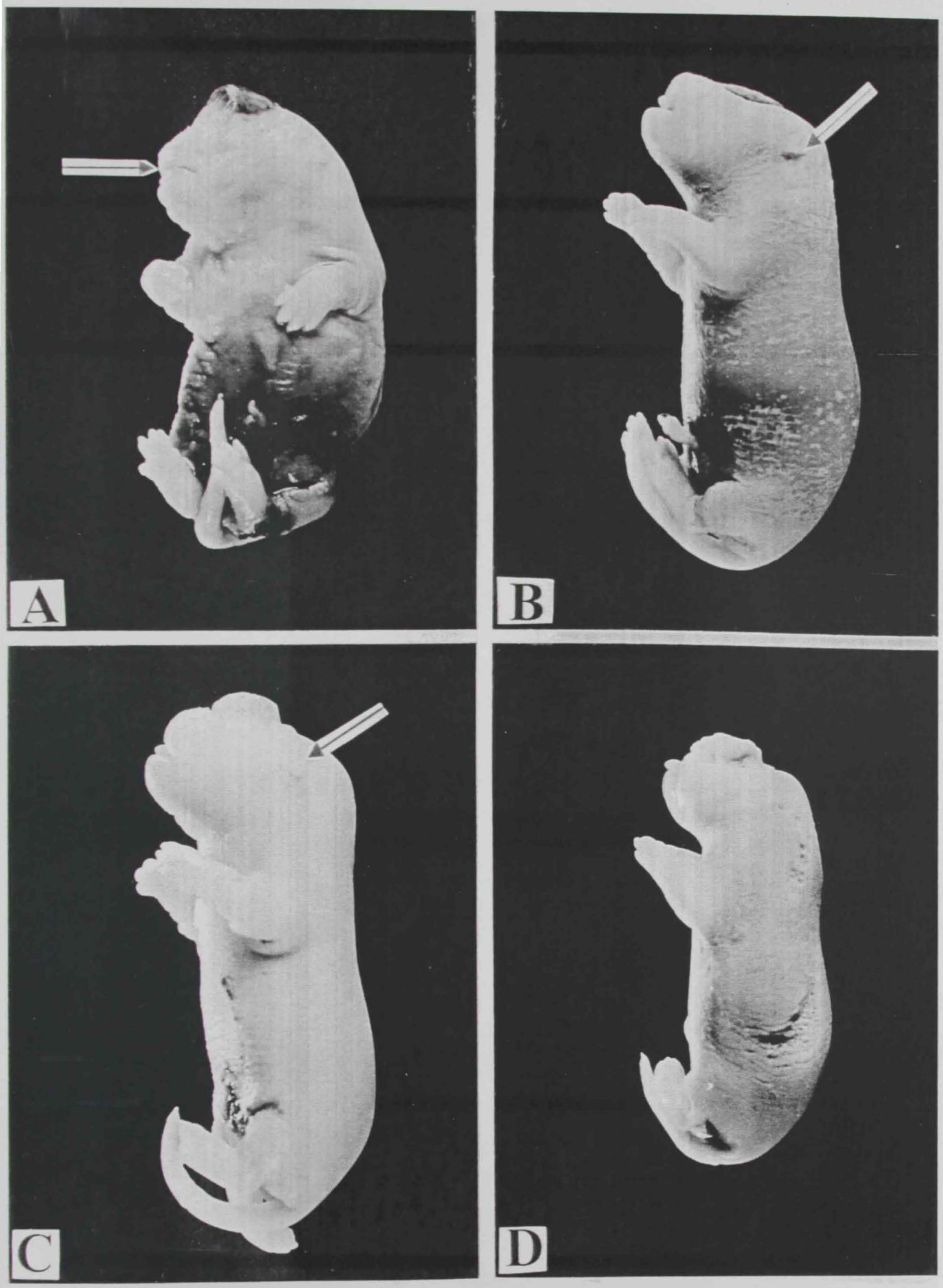
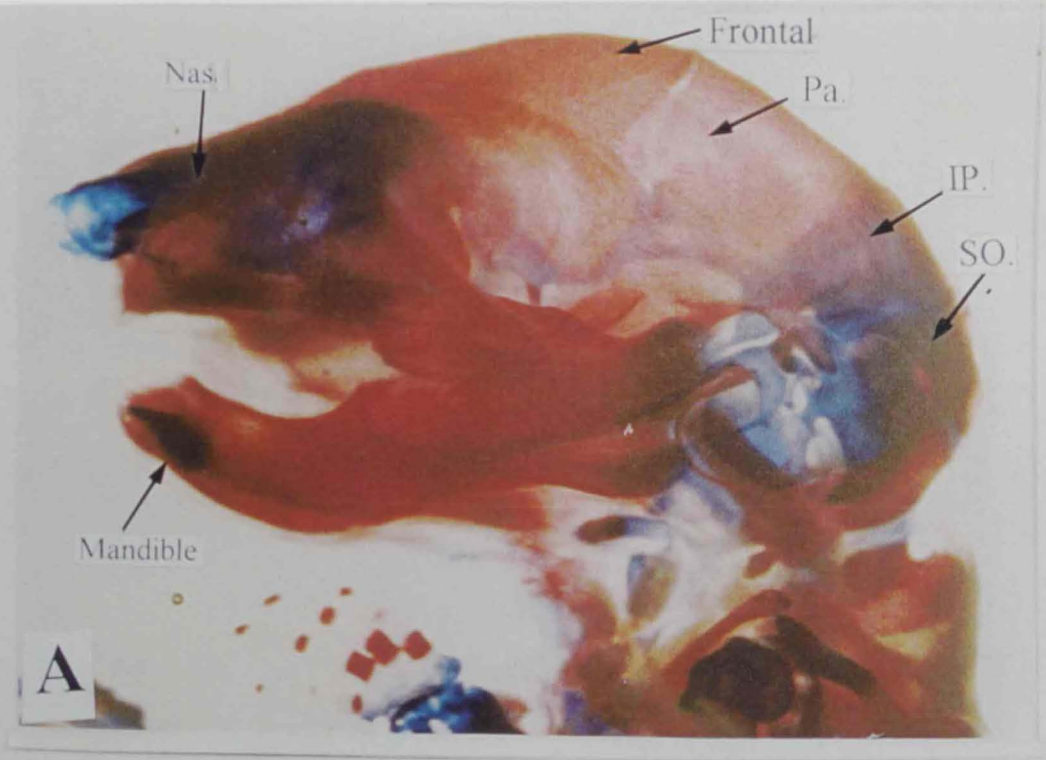

A
B
C
D

Fig 2 Anterior view of heat-stressed fetuses with edema, mandibular and maxillary hypoplasia and microtia Note also mandibular and maxillary hypoplasia (B, C), excencephaly (B, C, D) and growth retardation (D) Microtia is present in B, C. In the excencephalic fetuses, the exposed haemorrhogic brain tissue has degenerated. [A, C: 42°C/1hr on GD 9; B, D: 41°C/1hr on GD 9]

A
B

Fig 3 Lateral view of rat fetuses of gestation day (GD) 20. A: Growth retarded heat stressed fetus with microtia. In the excencephaly fetus (B), observe the facial cleft (arrow) and protrusion of the tongue. [A: 42°C/1hr on GD 9; B: 41°C/1hr on GD 9].


Frontal
Nas.
Pa.
IP.
SO.
Mandible
A


SO.
Meckle's
Cartilage
B

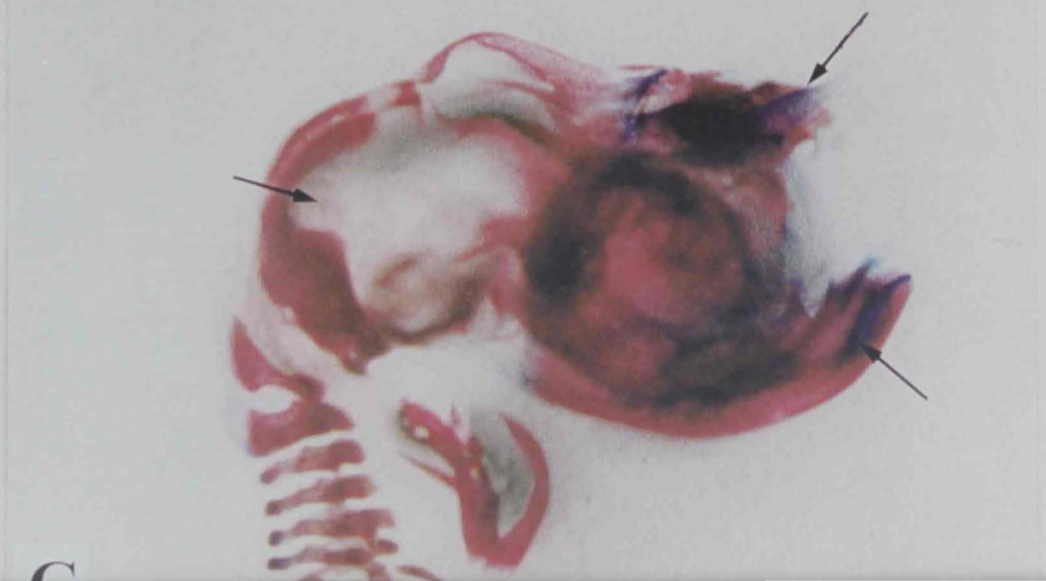

C

Fig. 4. Lateral view of skull bones of rat fetuses of gestation day (GD) 20. A: Control fetus showing well ossified skull bones. B: Heat stressed (42°C/1hr on GD 9) fetus whose skull bones are poorly ossified. Also the Meckle's cartilage is persistent. In exencephalic heat stressed (41°C/1hr on GD 9) fetus (C), maxillary hypoplasia is seen very well. Skull bones are poorly ossified. The nasal, frontal, parietal (Pa) and interparietal (IP) bones are absent. [SO: supra-occipital].

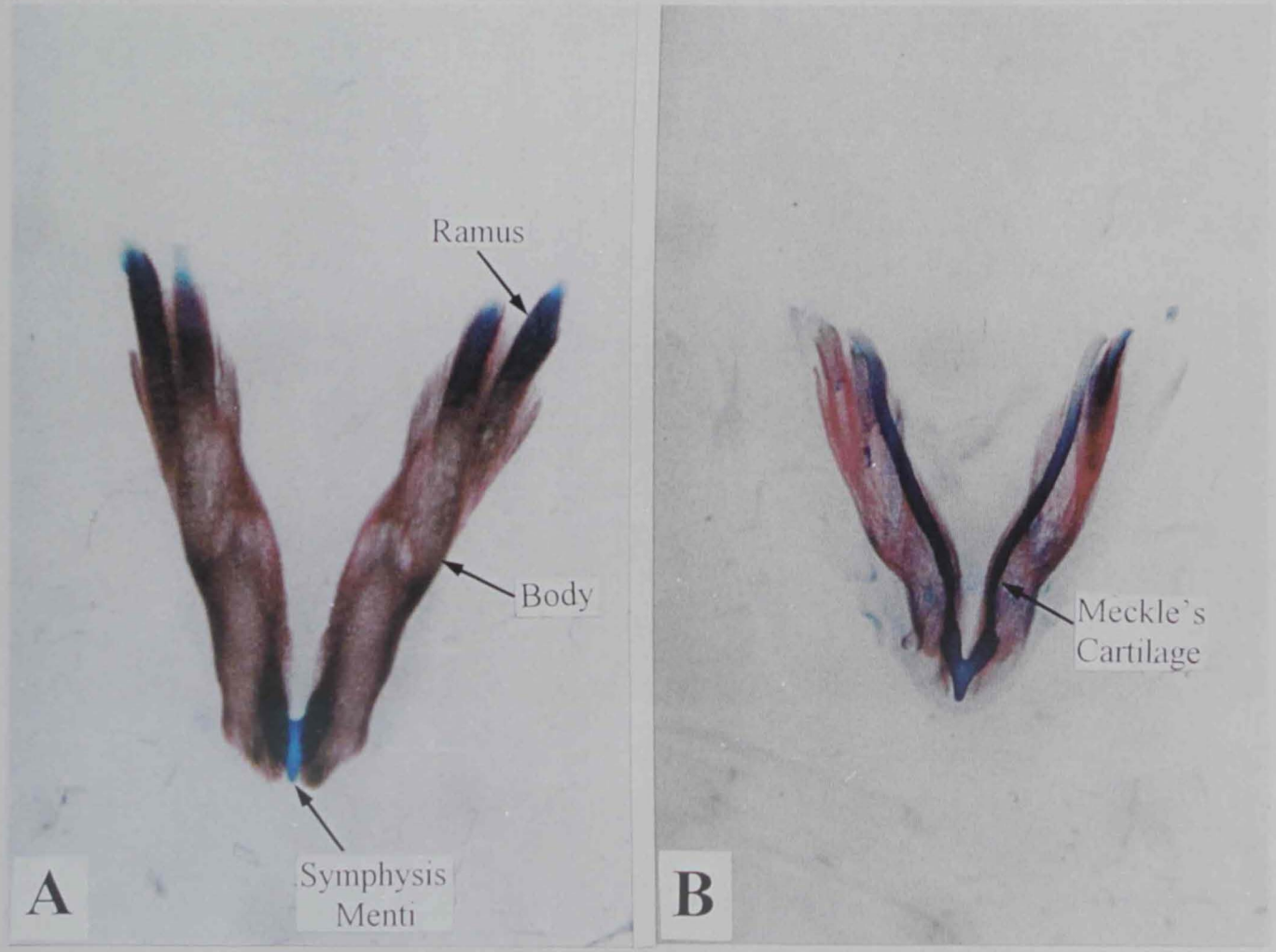

Ramus
Body
Symphysis
Menti
A
Meckle's
Cartilage
B

Fig. 5 Mandibles of rat fetuses of gestation day (GD) 20. A: The control fetus shows properly ossified body and ramus. In the heat stressed fetus (B) (42°C/1hr on GD 9) the mandible is remarkally hypoplastic and the entire Meckel's cartilage is persisting.

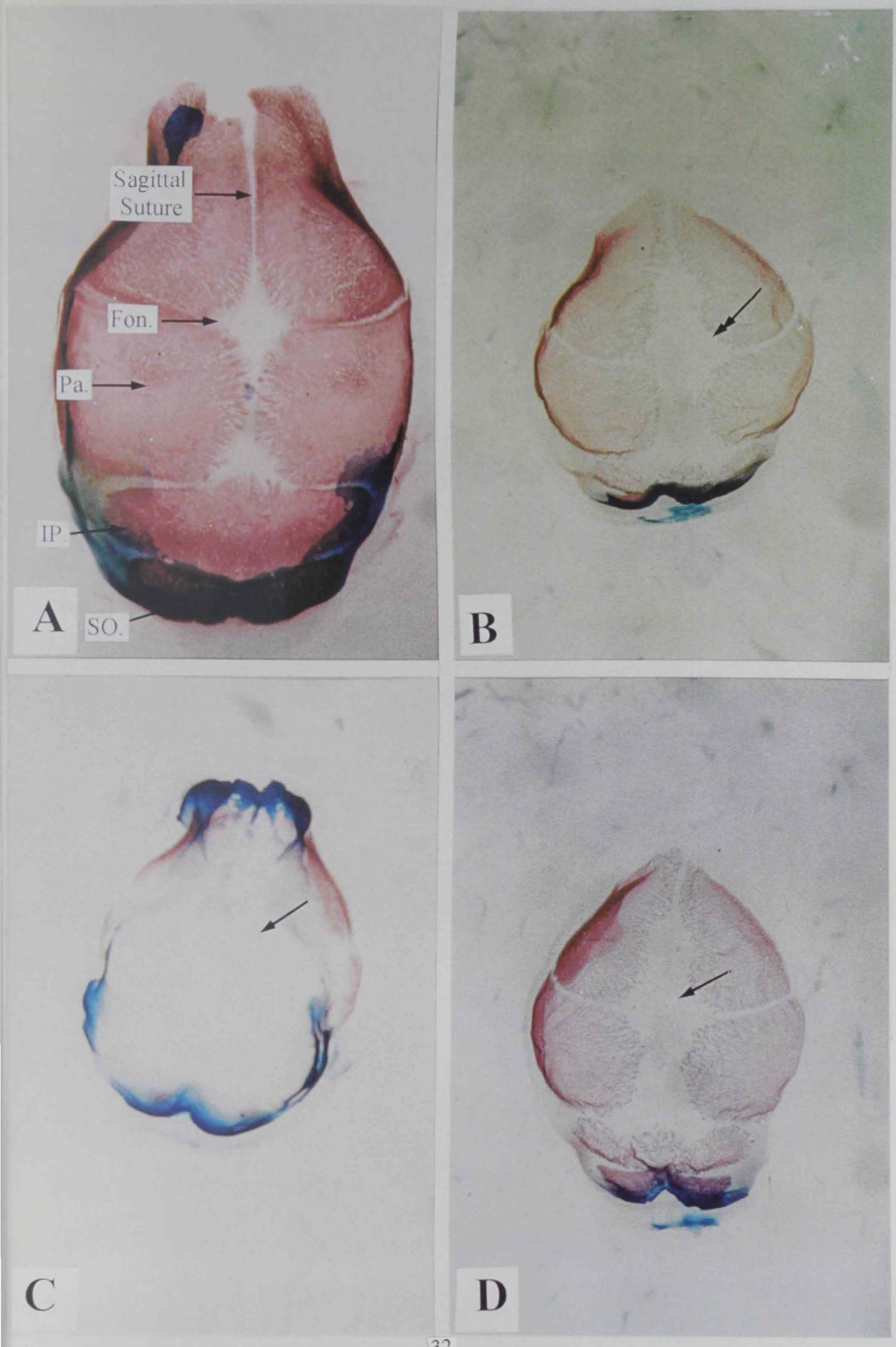

Sagittal Suture
Fon.
Pa.
IP.
SO.
A
B
C
D

Fig. 6. Skulls of rat fetuses of gestation day (GD) 20. A: The control fetus shows normal size of fontanelles and sutural lines. In the heat stressed (B: 41°C/1hr on GD 9; C, D: 42°C/1hr on GD 9) fetuses, the skulls are hypoplastic (B, C, D), sutures and fontanelles are wide (arrow in B, D) indicating poor ossification of the bones of the cranial vault. Fetus C shows only traces of ossification of the cranial vault. [Fon: fontanelle, Pa: Parietal bone, IP: inter-parietal bone, SO: supra-occipital bone].

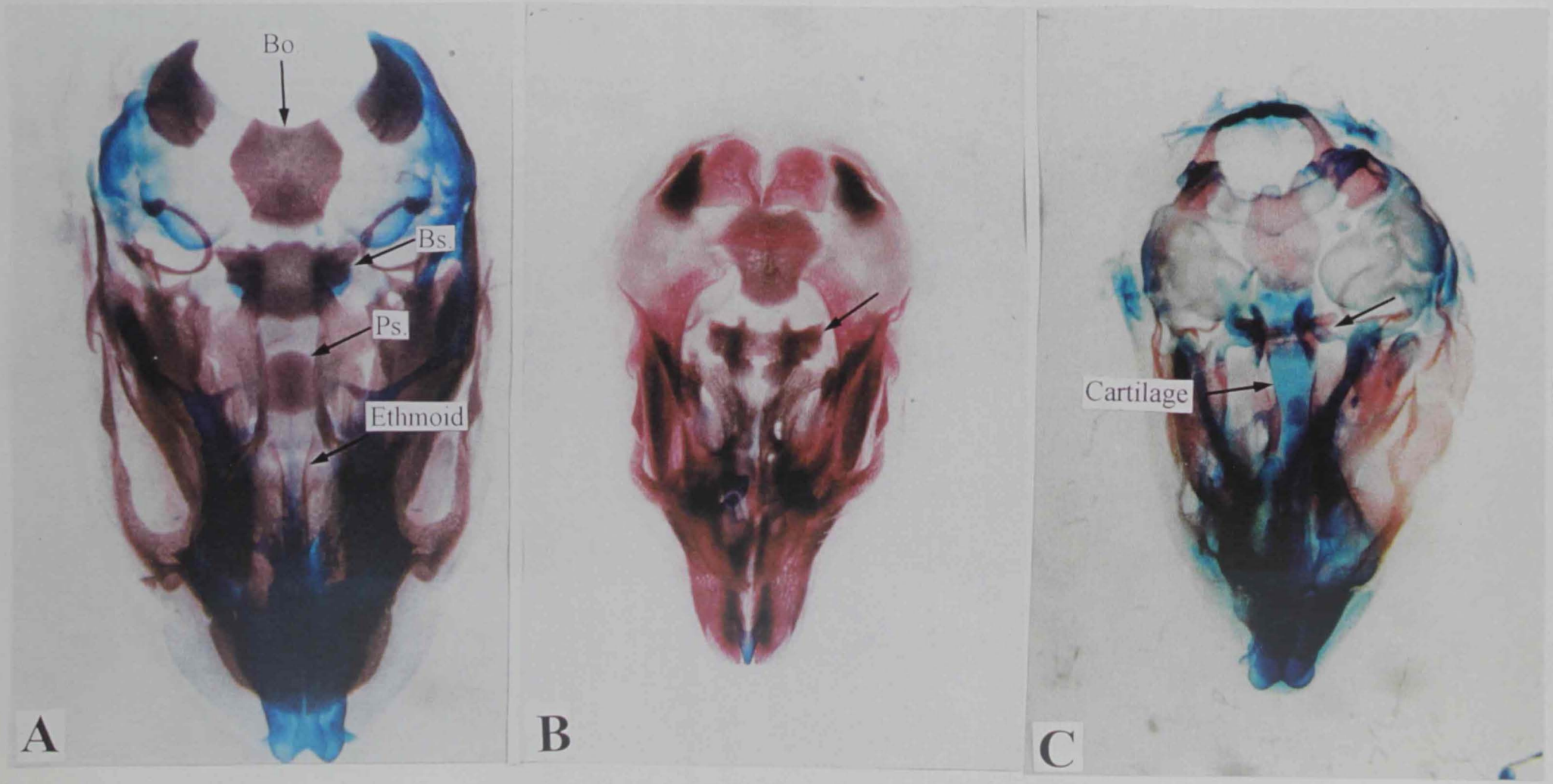

Bo
Bs.
Ps.
Ethmoid
A
B
Cartilage
C

Fig. 7. Basicrania of rat fetuses of gestation day (GD) 20. A: Control. The presphenoid (Ps), basisphenoid (Bs) and basioccipital (Bo) bones are well ossified. In heat stressed (41°C/1hr on GD 9) fetuses, the presphenoid is split into two haves (arrow in B) or split and hypoplastic (arrow in C) and the basisphenoid is hypoplastic. The ethmoid is absent in C. A median longitudinal cartilaginous bar (cartilage) in C indicates the unossified precursor of the median bones. Also observe the gross reduction in anteroposterior and transuser diameters of the basicranium (B).

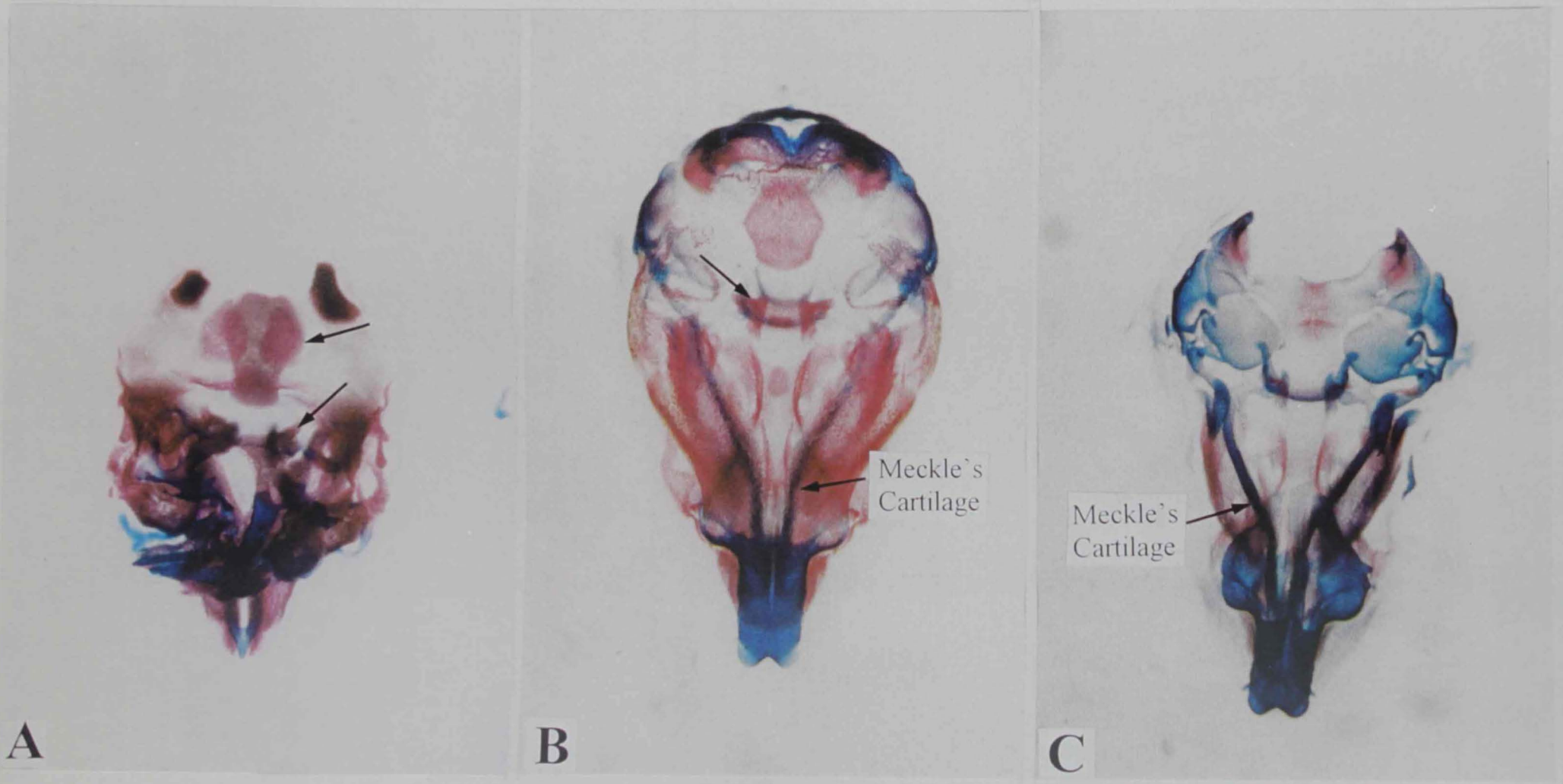

A
B
Meckle`s
Cartilage
Meckle`s
Cartilage
C

Fig 8 Basicrania of rat fetuses of gestation day (GD) 20. A: In heat stressed fetuses [A 41°C/1hr on GD 9; B, C: 42°C/1hr on GD 9] observe that the cranial vaults is missing and basicranial bones are crowded (A), hypoplastic (arrow in B), the Meckel's cartilage is persisting (arrow in B, C) and the tympanic ring is absent (A, C). Fetus A is an exencephalic one.

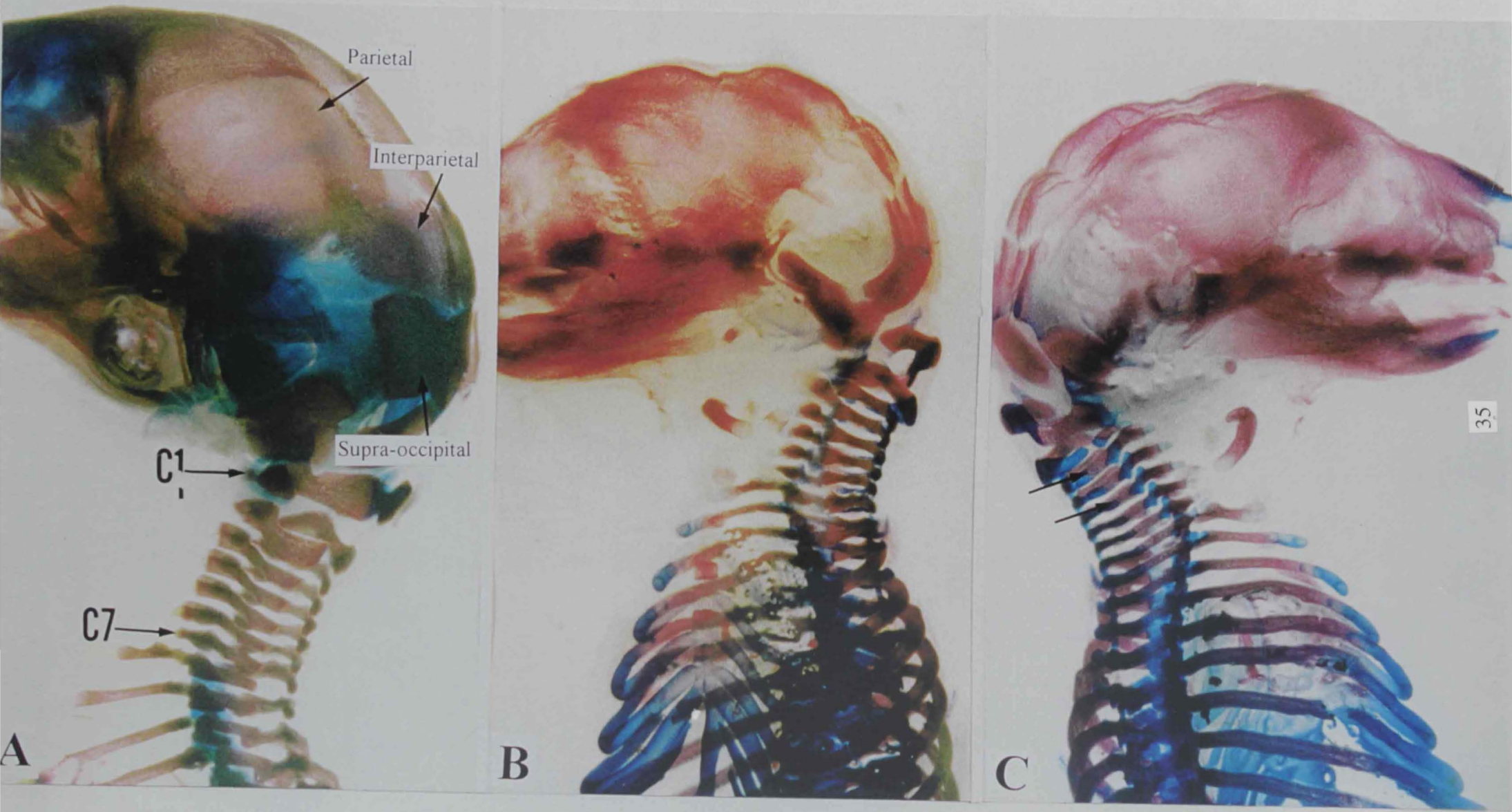

Parietal
Interparietal
Supra-occipital
C1
C7
A
B
C

Fig 9 Lateral view of the skulls and cervical region of rat fetuses of gestation day (GD) 20 A Control Observe the well-ossified skull bones and cervical vertebral arches (C1-C7) B, C The heat stressed (41°C/1hr on GD 9) fetuses show hypoplastic skull bones and cervical arches Note that the ossification of the arches are interrupted as indicated by the presence of cartilage (blue in B and C).

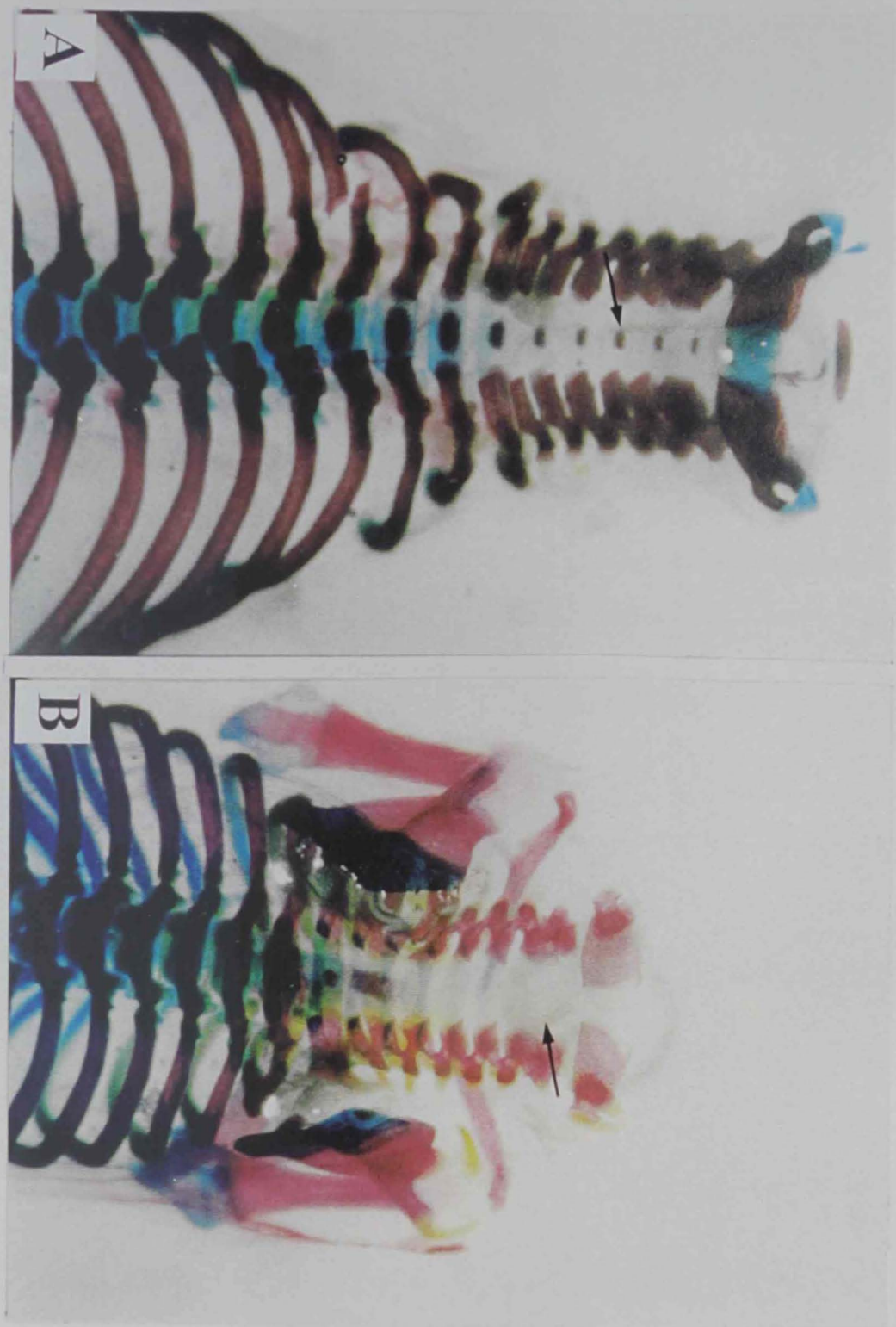

A
B

Fig 10 Rat fetuses of gestation day (GD) 20. A: The control fetus shows 6 vertebral bodies (arrow) and 7 arches in the cervical region, which are normally ossified. B: The heat stressed (41°C/1hr on GD 9) fetus has 7 cervical hypoplastic arches, the corresponding bodies (arrow) are unossified.

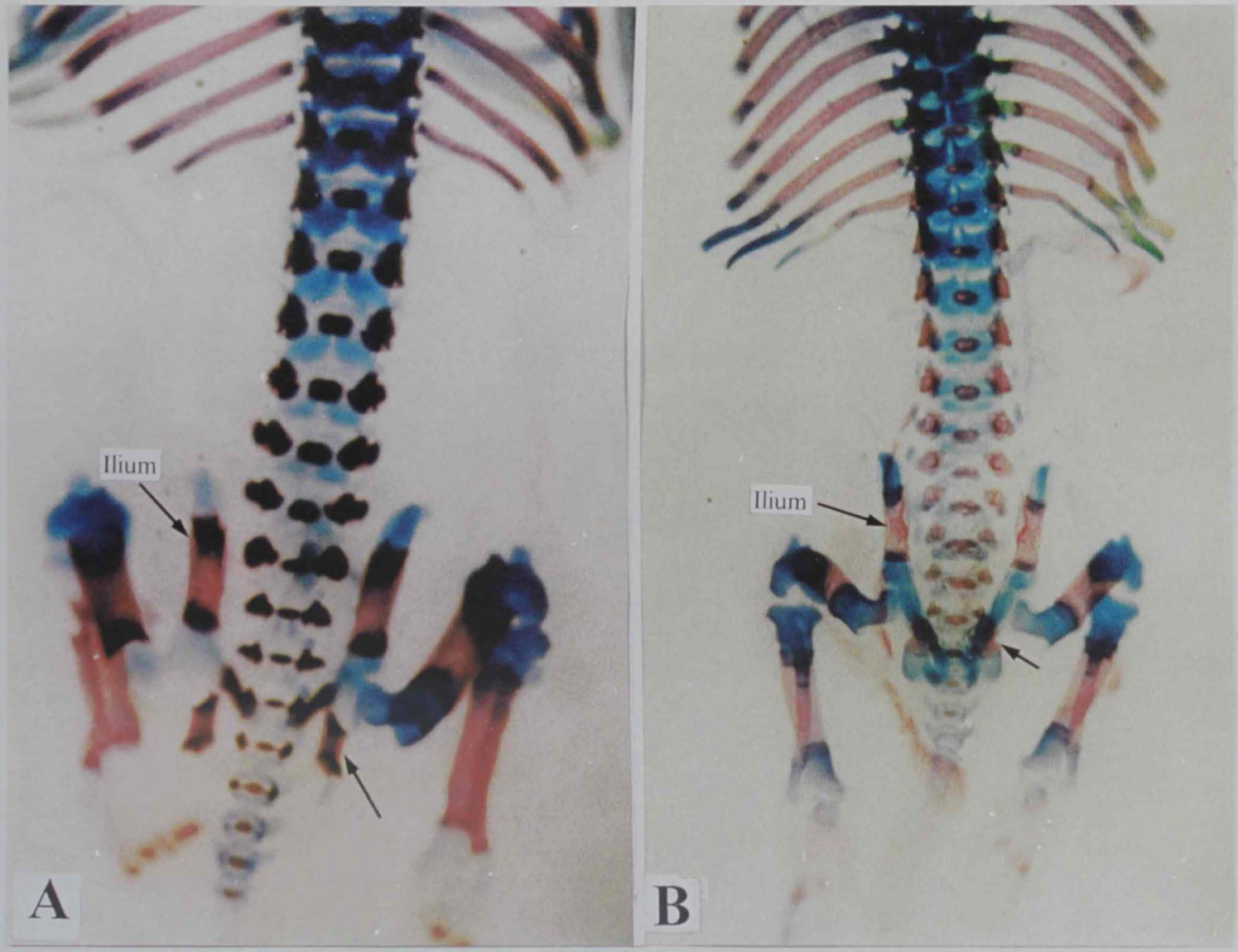

Ilium
A
Ilium
B

Fig 11 Dorsal view of fetal rat skeleton of gestation day (GD) 20 stained with alizarin red–S and alcian blue A The control fetus shows good ossification of the vertebrae of lumbosacral segments, pelvic girdle and the bones of the hindlimb. B: The heat stressed (41°C/1hr on GD 9) fetus shows poor ossification of the vertebrae of lumbosacral segments, pelvic girdle and the hindlimb.

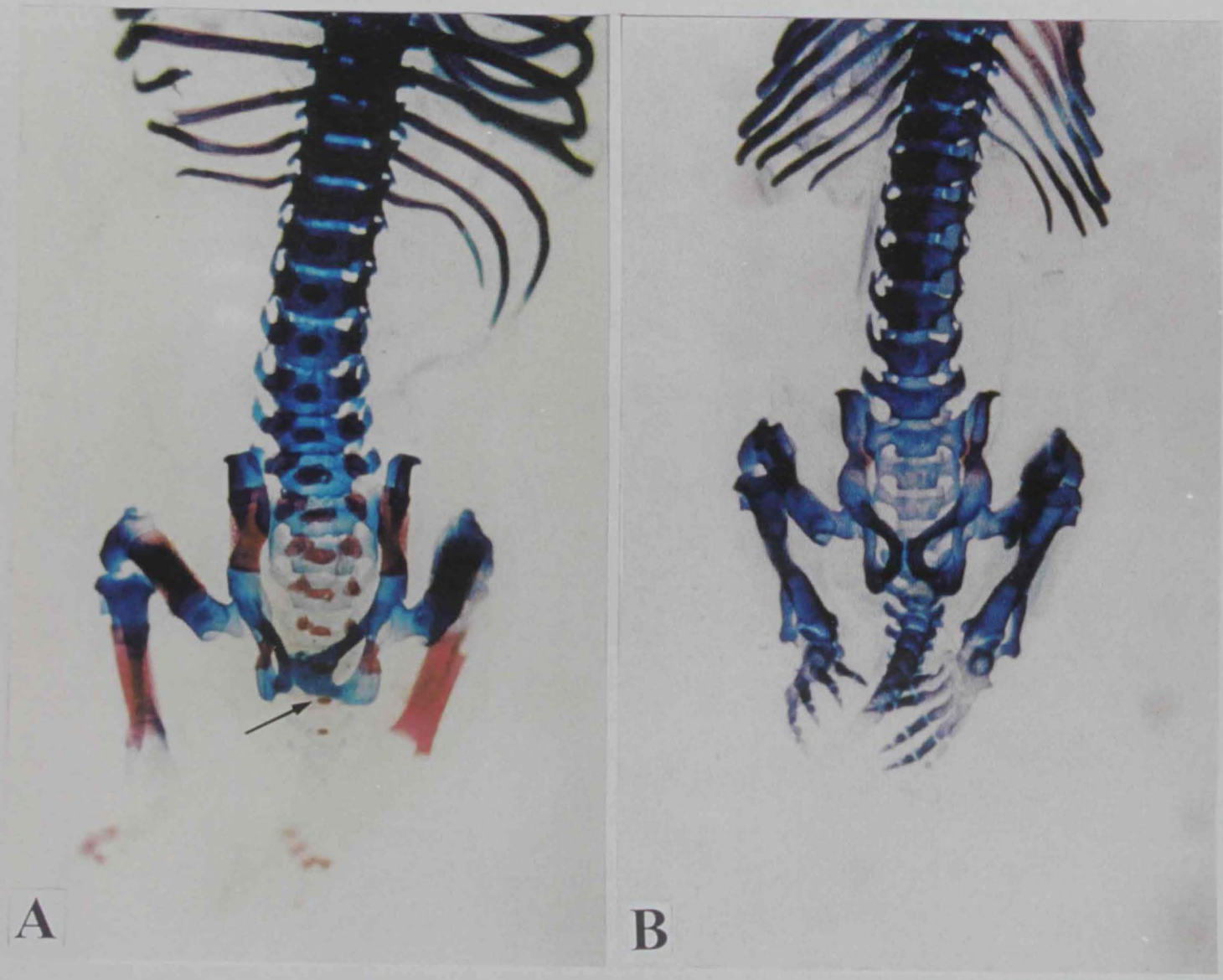
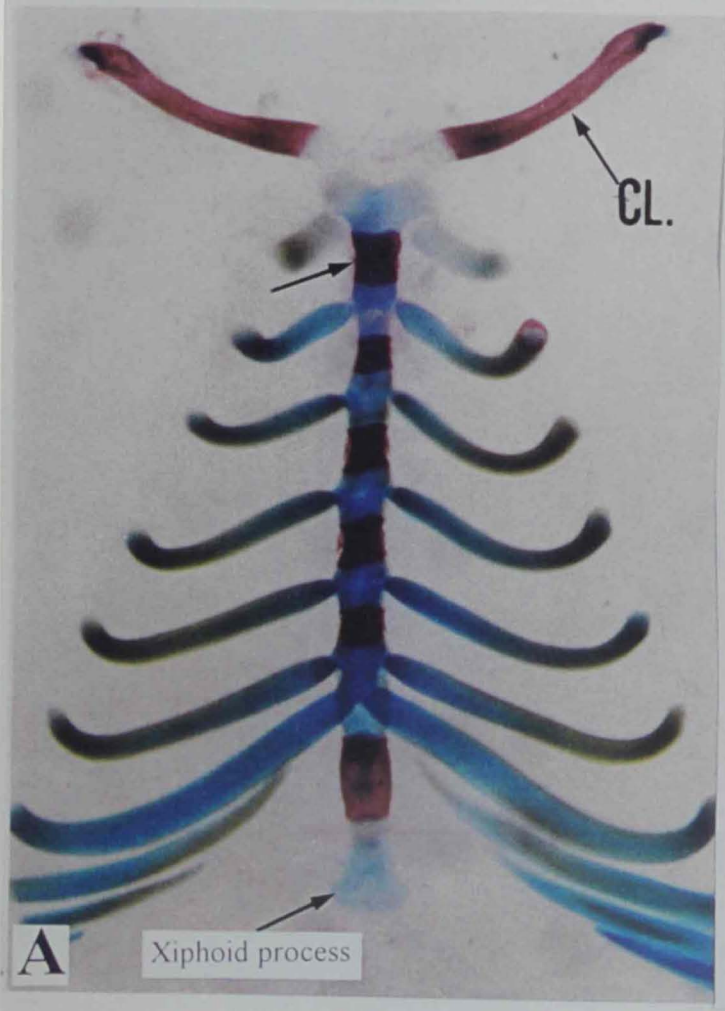
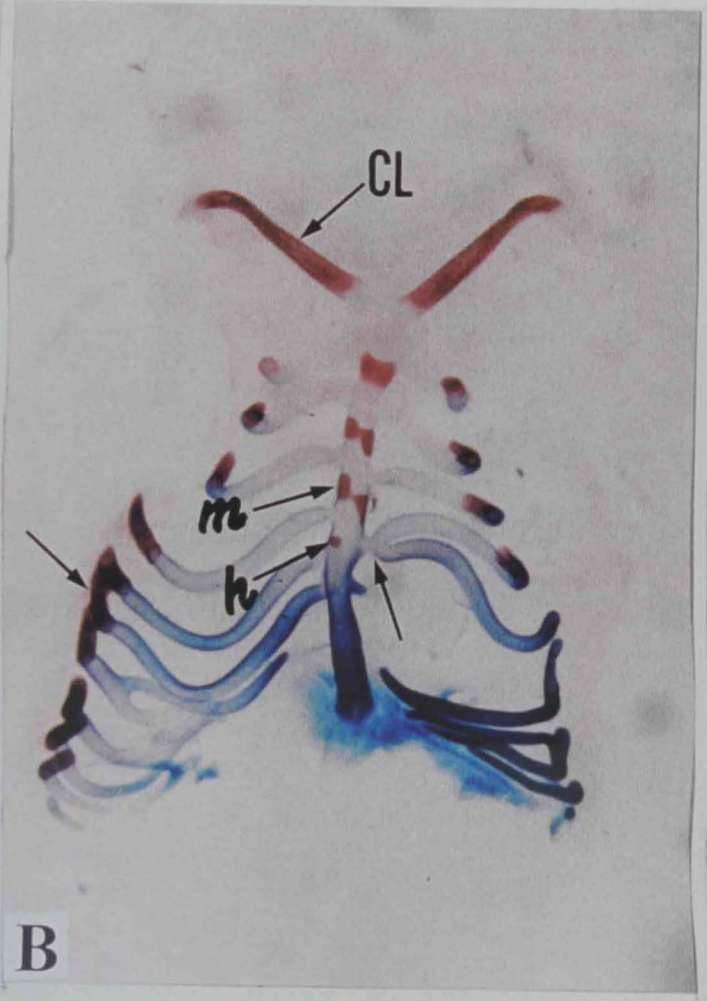

A
B

Fig 12 Dorsal view of fetal rat skeleton of gestation day (GD) 20 stained with alizarin red–S and alcian blue. A: The coccygeal segment shows 2 ossified bodies and arches (arrow in A). Fetus B, exposed to a higher temperature (42°C/1hr on GD 9) is extremely growth retarded and shows a virtual lack of ossification.


CL.
Xiphoid process
A


CL
m
h
B

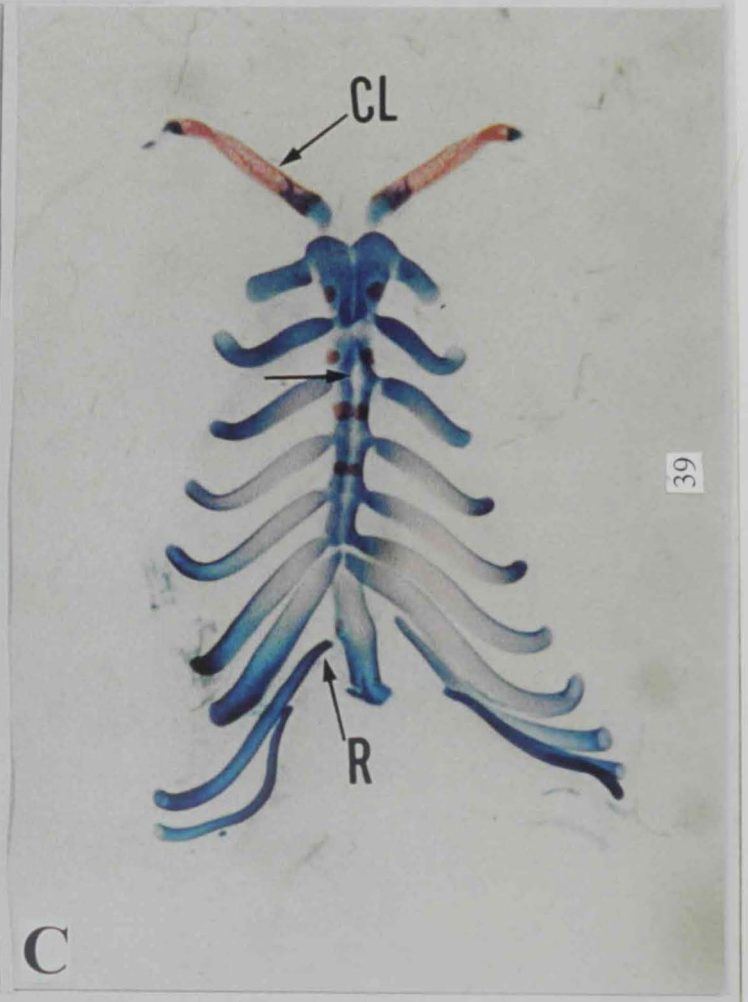

CL
R
C

Fig. 13. Skeleton of the ventral chest wall of rat fetuses of gestation day (GD) 20 stained with alizarin red-S and alcian blue. A: Control. The sternum has 6 sternebrae with 7 pairs of ribs attached to it. In fetus B, there are only 4 poorly ossified sternebrae, ribs 5, 6, 7 on the right side are fused (arrow), ribs 4, 5 of the left side are fused at their sternal end (arrow) and rib 6 is absent. Clavicales (CL) are short and hypoplastic (B, C). In fetus C, the sternebrae are poorly ossified and remain unfused (arrow in C), thus forming a "split-sternum". The rib 8 (R in C) is close to xiphoid process (arrow in C). m: malaligned sternebrae, h: hemisternebra. [B, C: 41°C/1hr on GD 9].

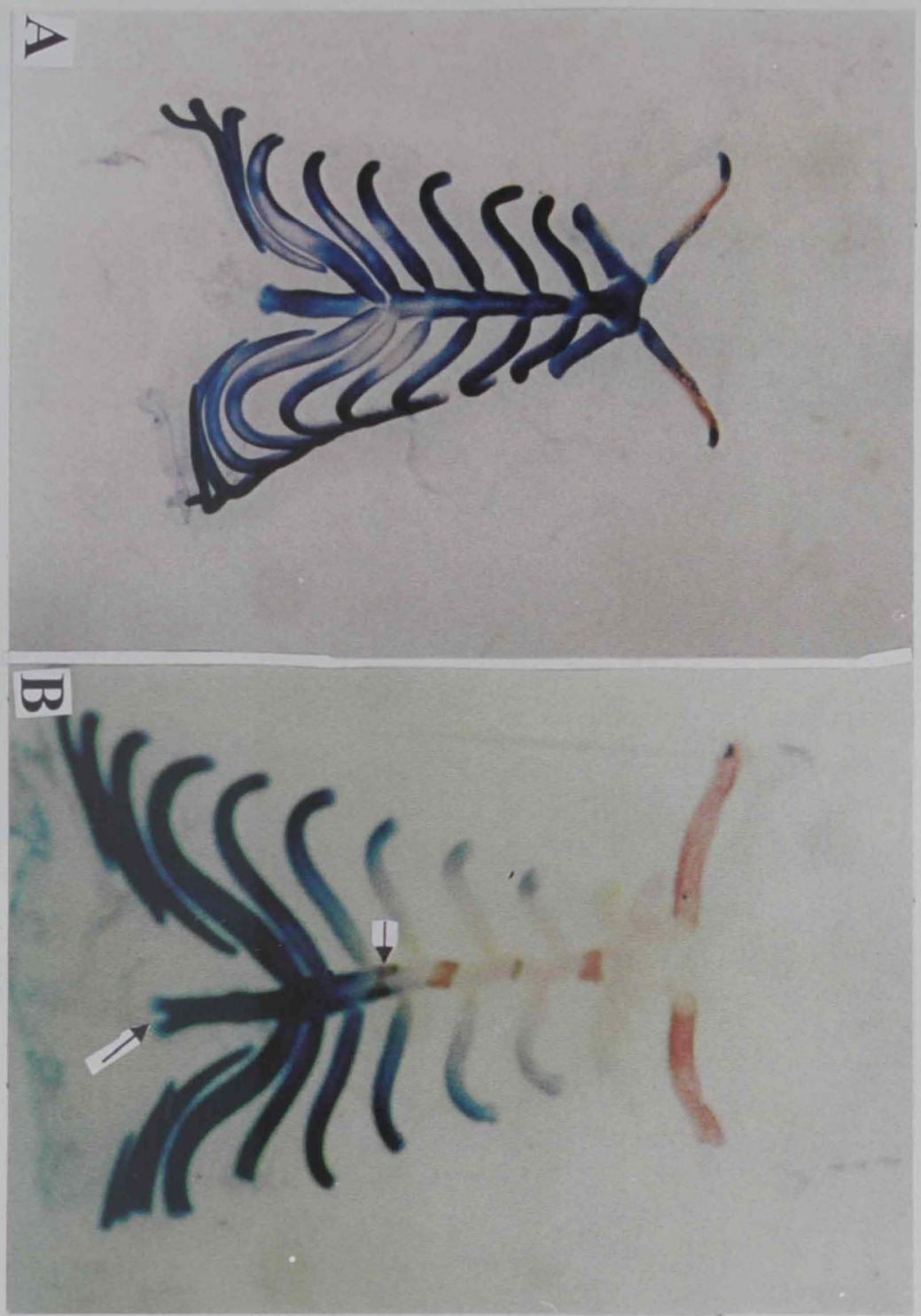
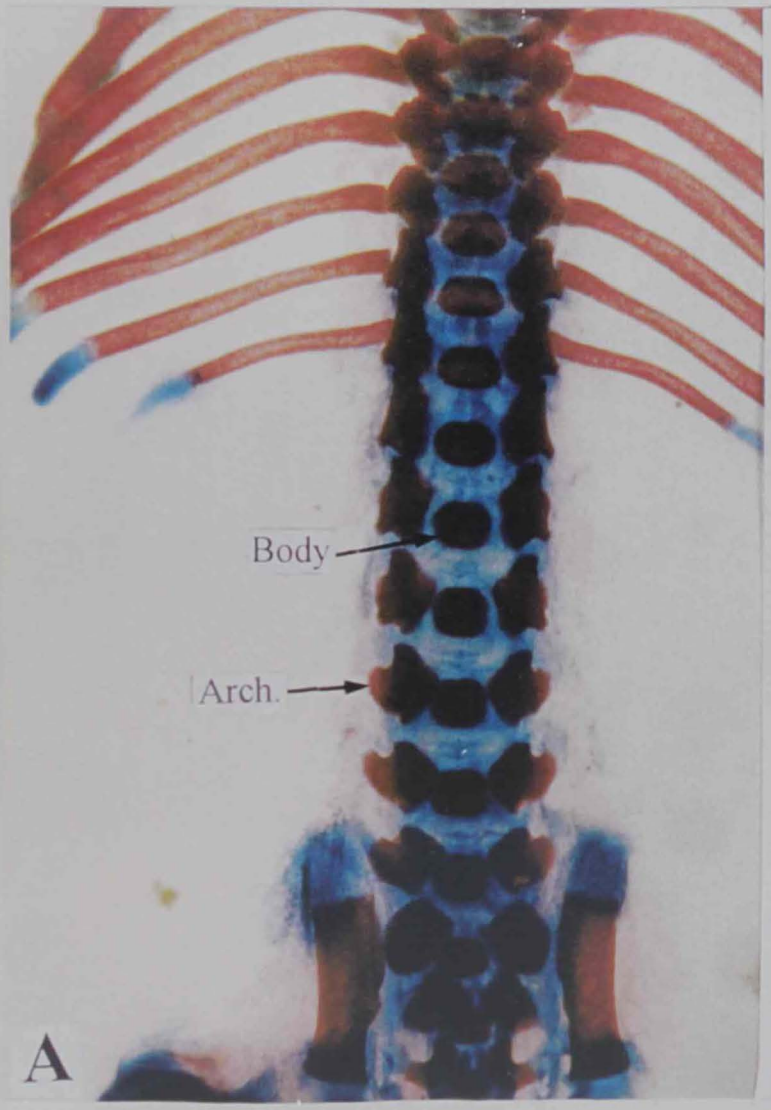

A
B

Fig. 14. Skeleton of the ventral chest wall of rat fetuses of gestation day (GD) 20 stained with alizarin red-S and alcian blue. In these heat stressed fetuses [A: 42°C/1hr on GD 9; B: 41°C/1hr on GD 9], the sternal bars are split and unossified (A) or poorly ossified (B). Fetus B has poorly ossified ribs on both sides and 4 sternebrae which are hypoplastic, malaligend and split (short arrow). Xiphoid process is also split (B) (long arrow).


Body
Arch.
A

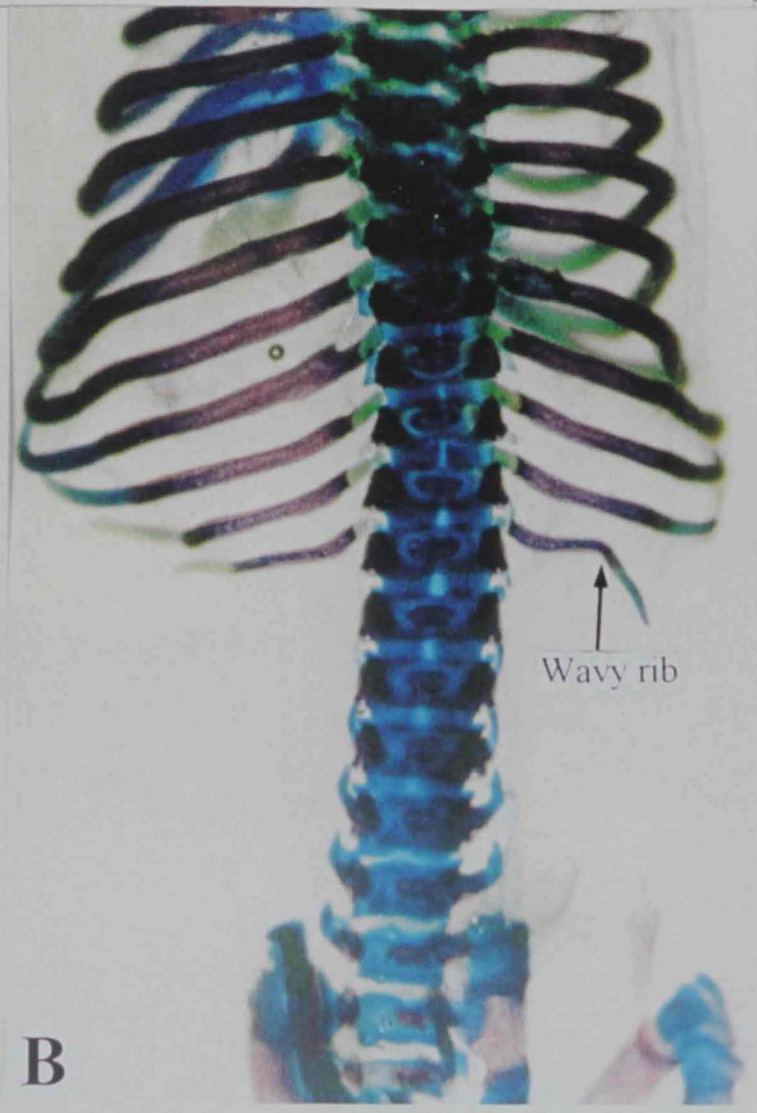

Wavy rib
B

Fig 15 Dorsal view of the lower thoracic and lumber segments of the axial skeleton of rat fetuses of gestation day (GD) 20. A: The control fetus shows very well ossified thoraco-lumber segment. B: Observe in the heat stressed (41°C/1hr on GD 9) fetus the 13^{th} rib is wavy.

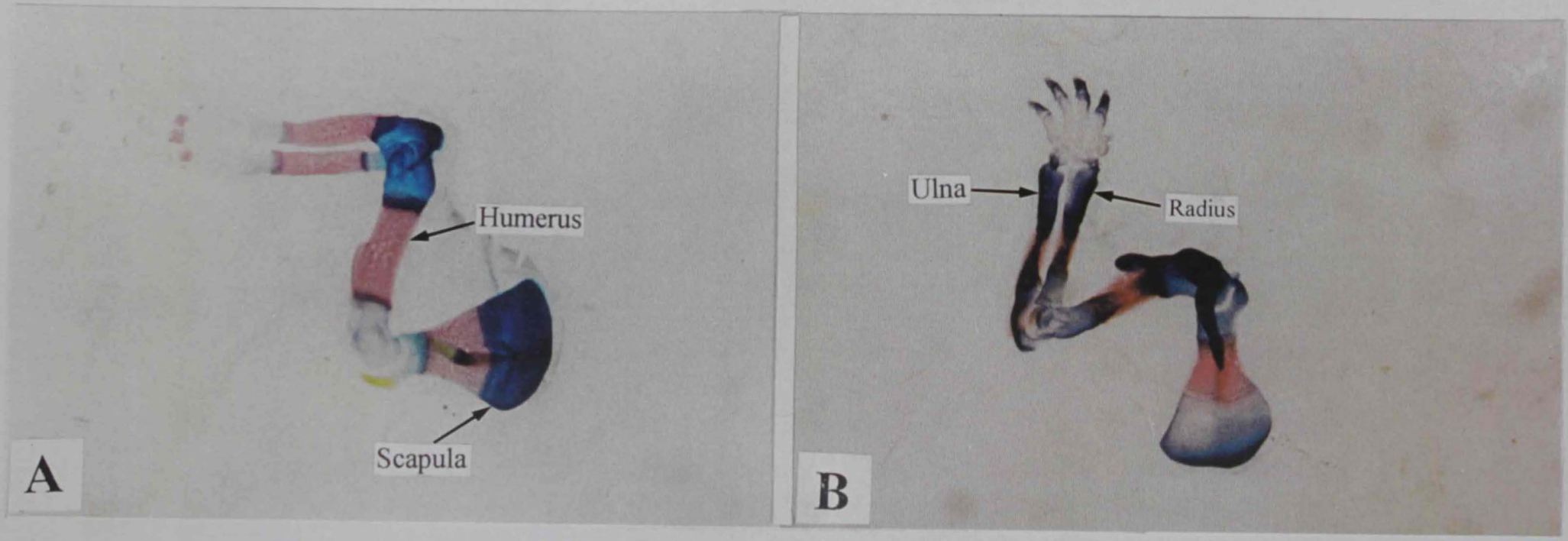

Humerus
Scapula
A
Ulna
Radius
B

Fig 16 Forelimb skeletons of rat fetuses of gestation day (GD) 20 stained with alizarin red-S and alcian blue. A: Control. B: Heat stressed (42°C/1hr on GD 9) fetus showing poorly ossified scapula, ulna and radius.

A
DB
BZ
LZ
B
GC
BA
Glc
C
EB
GC
BA
D
GC

Fig 17 Sections of control rat placentae of gestation day 20. A, B: Note the three zones of the placenta: the deciduas basalis (DB), basal zone (BZ) and labyrinthine zone (LZ). The deciduas basalis comprises fibroblast like cells (FB in C) and collagen fibres. The basal zone presents giant cells (GC in C, D) and basophils (BA in C). A, 10X, B, 20X; C-D, 40X.

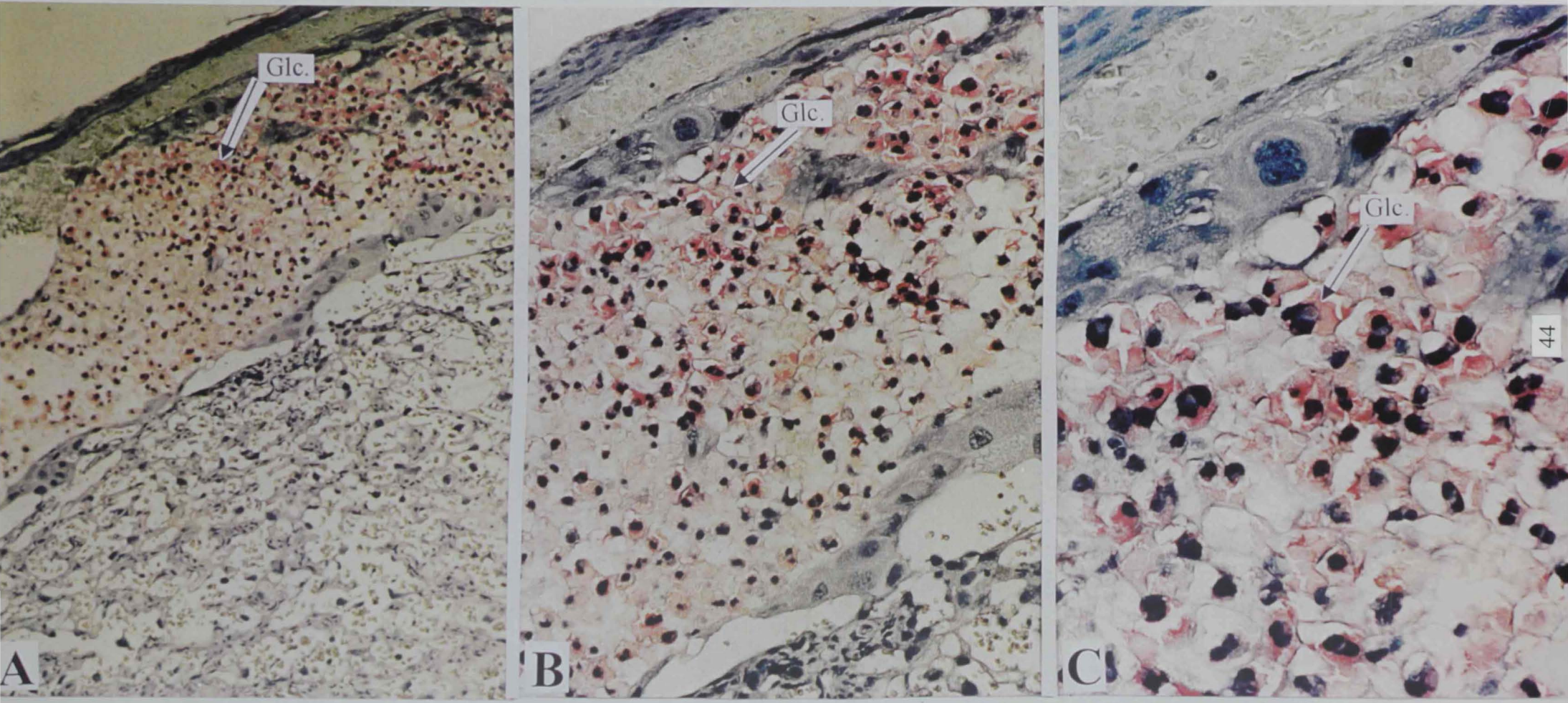

Glc.
Glc.
Glc.
A
B
C

Fig 18. Placentae of control rat fetuses of gestation day (GD) 20 stained with Best's carmine. Observe the glycogen (Glc) cells, that are abundant in the basal zone and appear red in color (A, B, C). A, 10X; B, 20X; C, 40X.

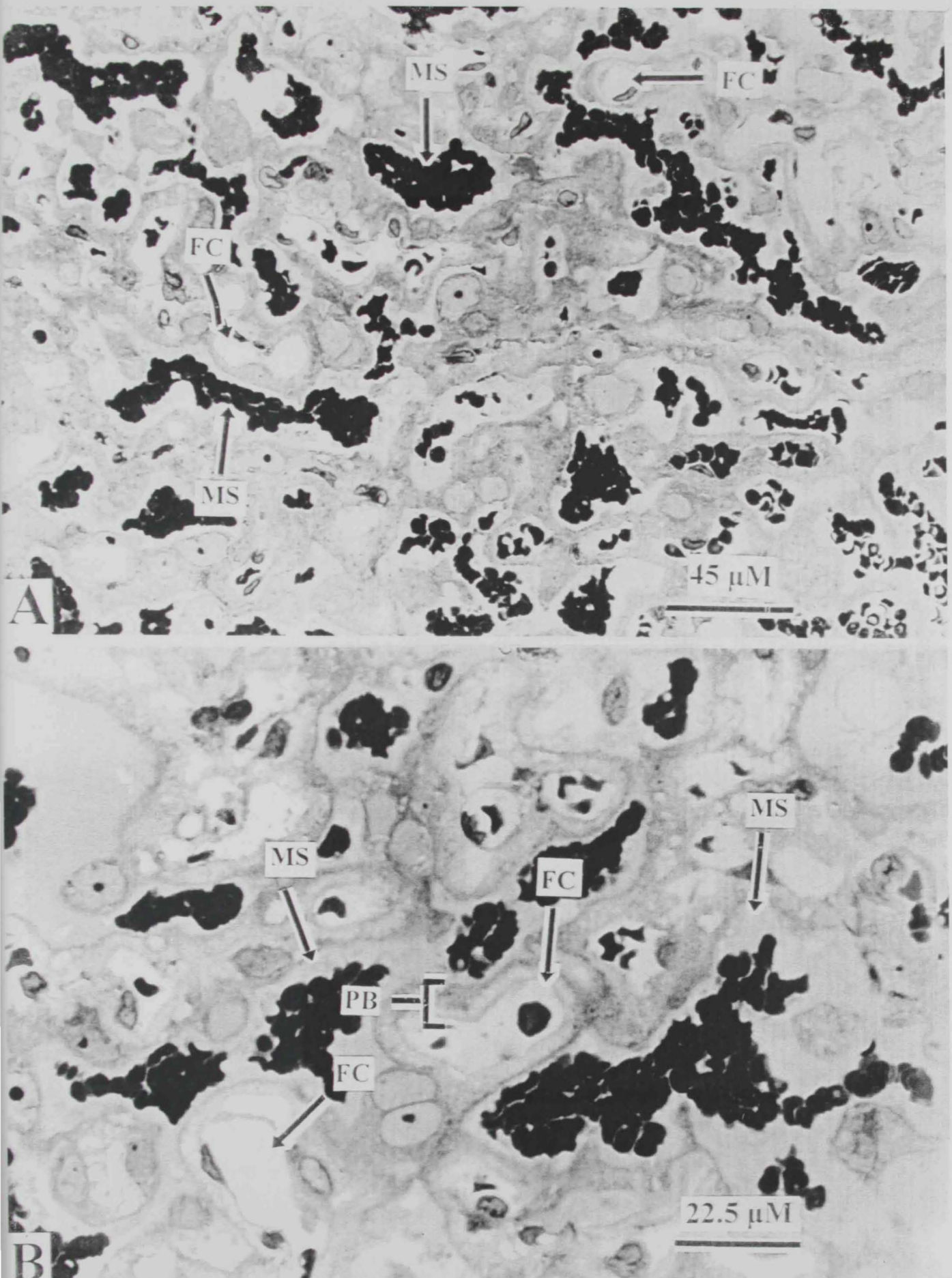

MS
FC
FC
MS
45 µM
A
MS
FC
MS
PB
FC
22.5 µM
B

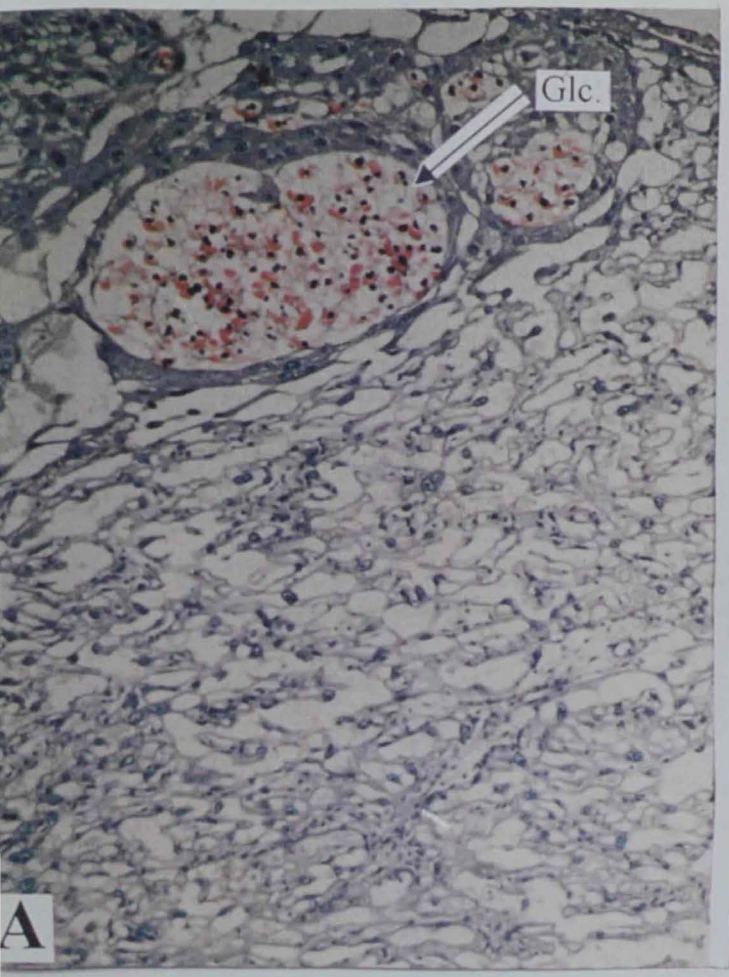
Fig 19 Sections of placentae of rat fetuses of gestation day (GD) 20 A: Section of a control placenta showing the labyrinthine zone (LZ) with finger like labyrinths with intervening maternal sinusoid (MS). The fetal capillaries (FC) are separated from the MS by three layers of trophoblast (B) PB placental barrier

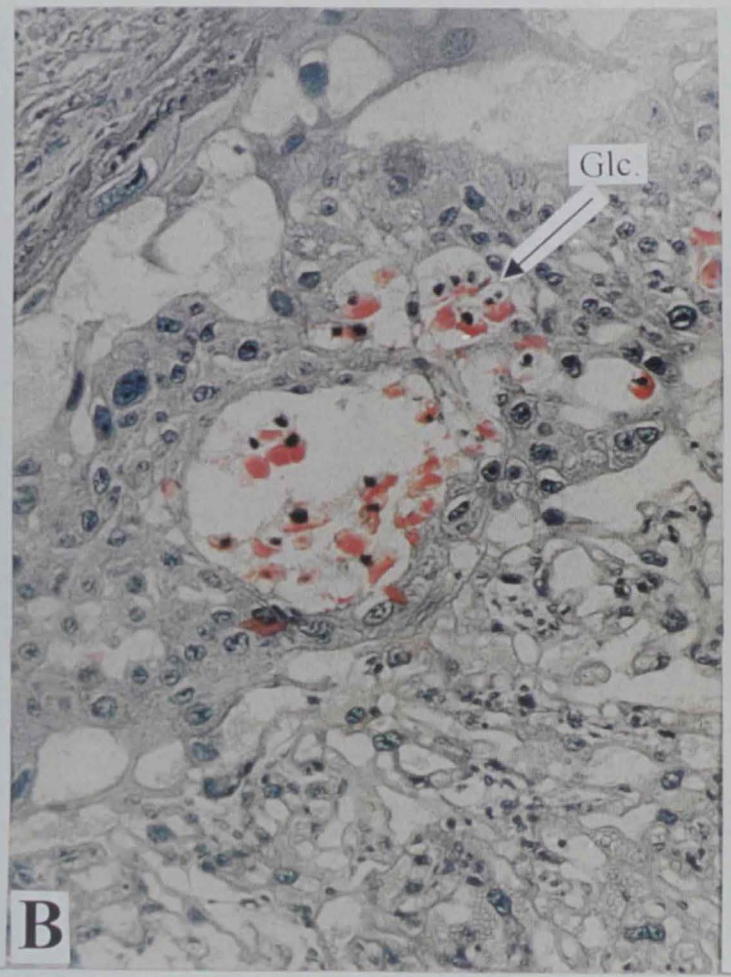
A
DB
BZ
LZ
B
C
DB
D
GC

Fig 20 Sections of heat stressed (41°C/1hr on GD 9) placnetae of rat fetuses of gestation day (GD) 20 A, B Section of deciduas basalis (DB), basal zone (BZ) and labyrinthine zone (LZ) The decidua basalis is hyalininzed (C), the basal zone appears vacuolated (B) and the giant cells have proliferated (GC in D) A, 10X, B, 20X, C-D, 40X

A
Glc.
B
Glc.
C
Glc.

Fig 21. Placentae of rat fetuses of gestation day (GD) 20 stained with Best's carmine. The sections of the heat stressed (41°C/1hr on GD 9) group show carmine positive glycogen (Glc) cells which are fewer in number than those of the control in the basal zone (A, B, C). A. 10X; B, 20X; C, 40X.


Glc.
A


Glc.
B

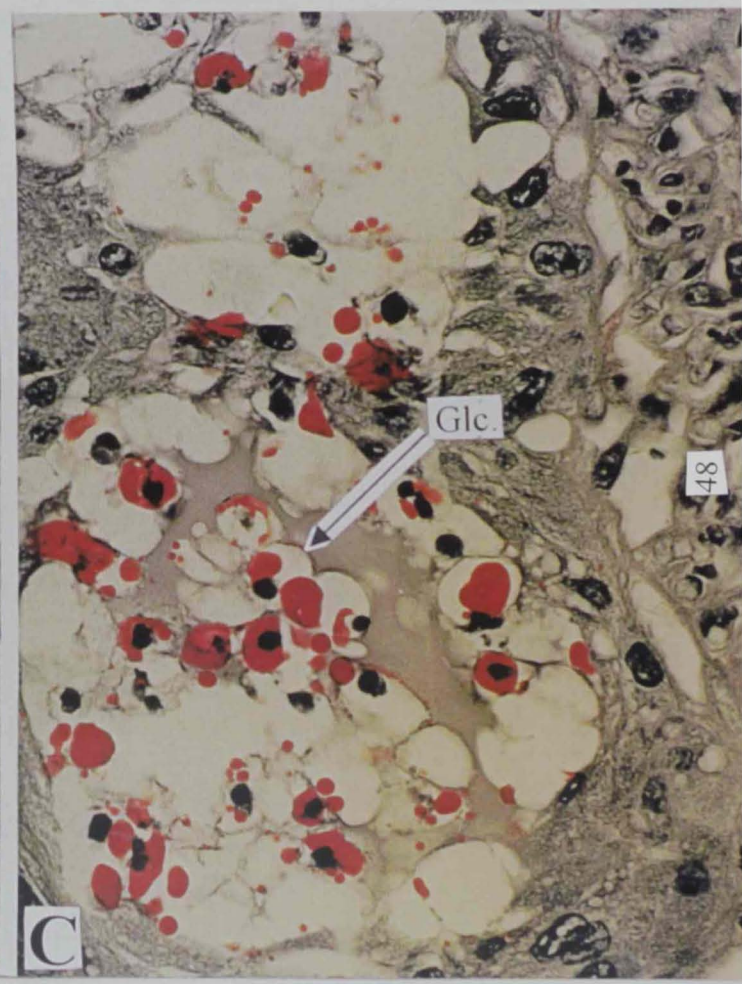

Glc.
C
48

Fig 22 Placentae of rat fetuses of gestation day (GD) 20 stained with Best's carmine The animals were subjected to a higher temperature (42°C/1hr on GD 9) The sections show glycogen (Glc) cells which are fewer in number than those of the control (Fig 23) and appear megenda red in color in the basal zone (A, B, C). A. 10X, B, 20X; C, 40X

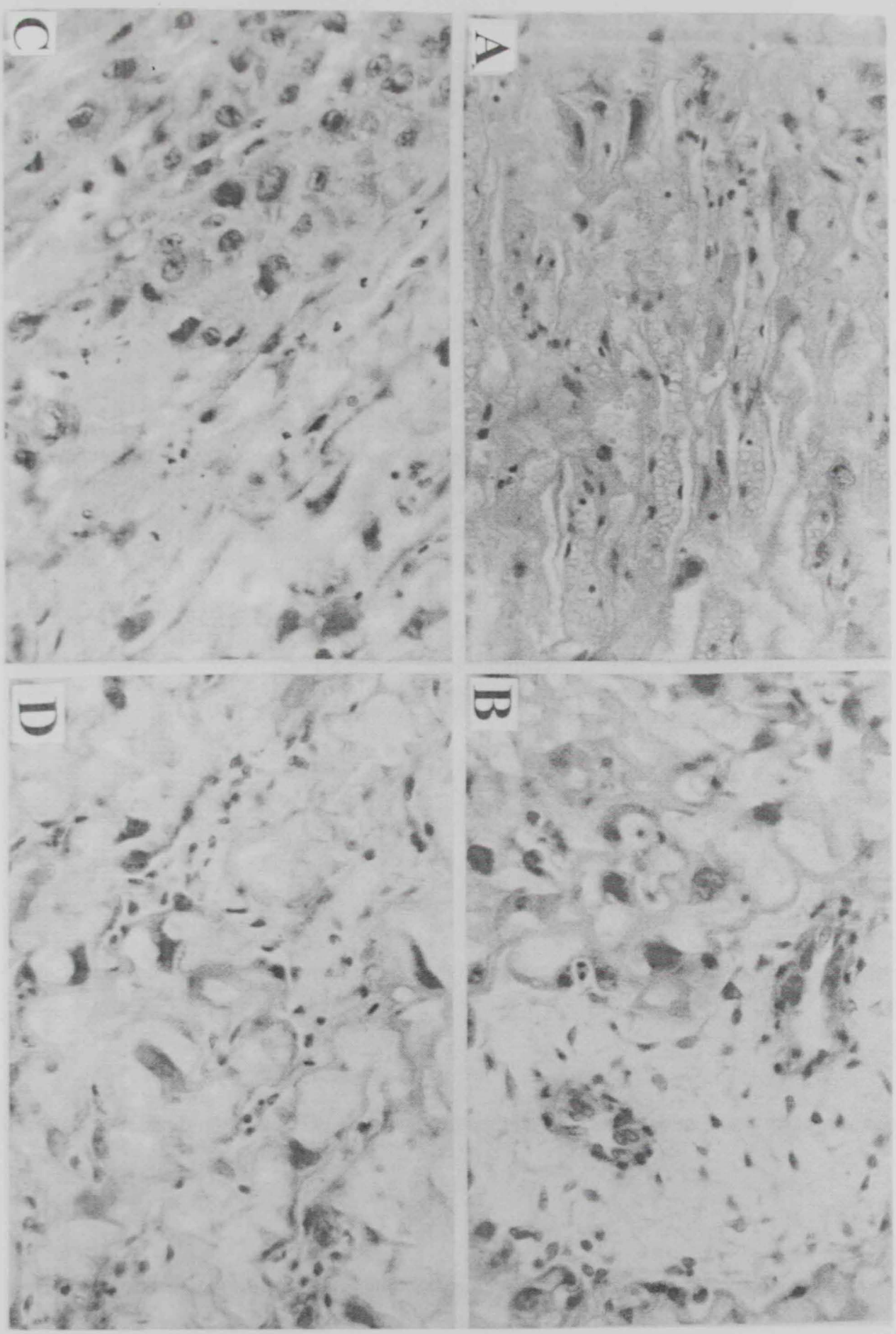

C
A
D
B

Fig. 23. Sections of placentae of rat fetuses of gestation day (GD) 20. A: Heat stressed (41°C/1hr on GD 9) section showing partly hyalinized labyrinthine zone (A, C), perivascular mesenchyme (B) that contributes to thickening of the placental barrier. Distortion of the cytoarchitecture and paucity of vascular development in the labyrinthine zone are obvious in D. Note also the presence of abundant giant cells in the basal zone in C. A-D, 40X. [A: 41°C/1hr on GD 9; B, C: 42°C/1hr on GD 9].

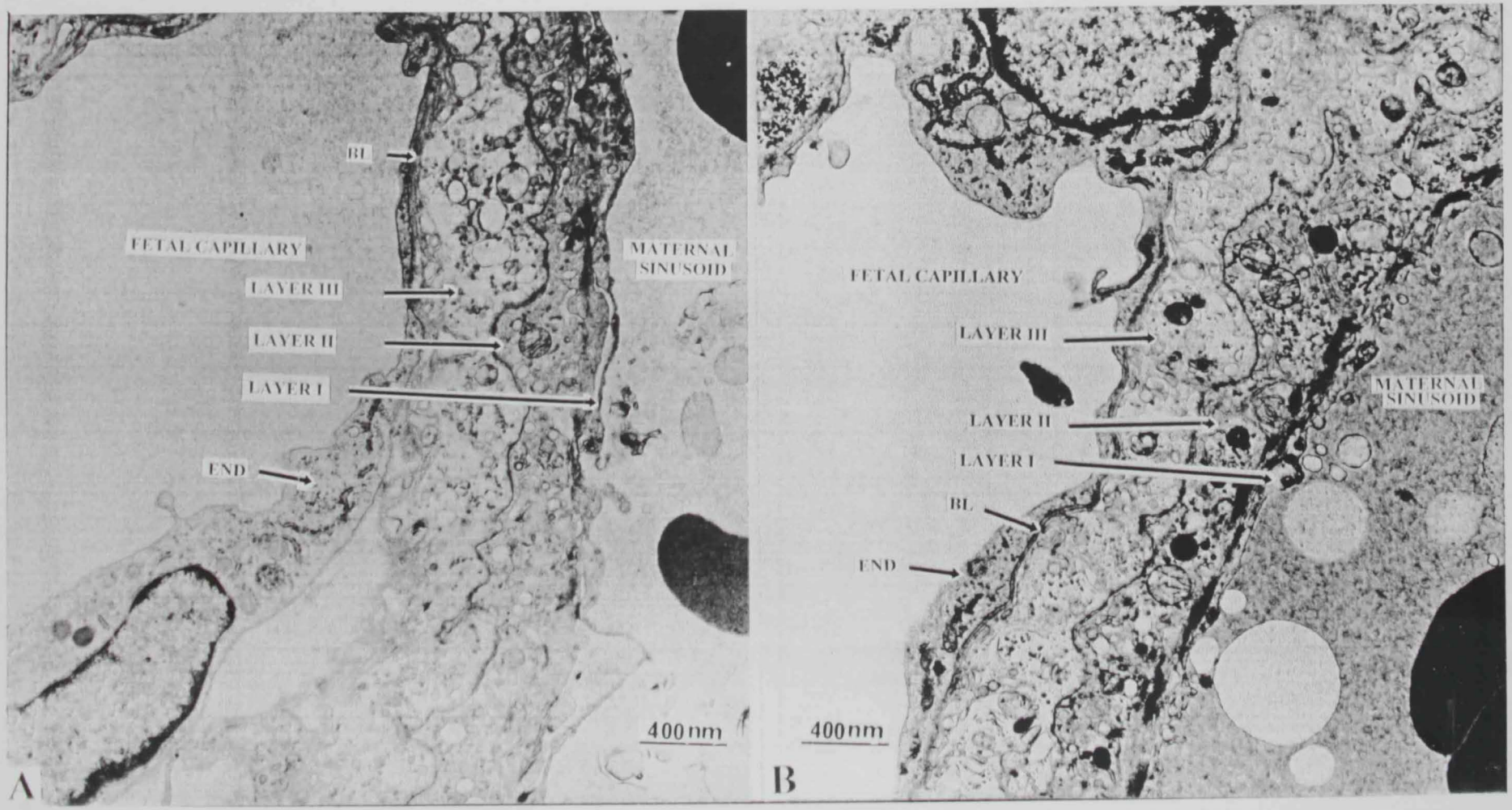

BL
FETAL CAPILLARY
MATERNAL SINUSOID
LAYER III
LAYER II
LAYER I
END
400nm
A
FETAL CAPILLARY
LAYER III
MATERNAL SINUSOID
LAYER II
LAYER I
BL
END
400nm
B

Fig. 24. Electron micrographs of the placentas of rat a fetuses of gestation day (GD) 20 showing the placental barrier in the labyrinthine zone. A: Control; B: Experimental. The fetal capillary blood is separated from the maternal sinusoid (MS) by the barrier composed of the trichorial membrane and the capillary wall. The trichorial membrane consists of three trophoblast layers: layer I, II and III (B). Note the numerous tight junctions between layer II and III. Layer I contains numerous pinocytic vesicles and shares a common basal lamina (BL) with the capillary endothelium (END in A and B). Observe the relative reduction in number of pinocytic vesicles in layer I, the presence of numerous electron dense granules in layer II and fibrinoid substance of in the peripheral cytoplasm of layer II. Bar = 400 nm.

Table 2. Effect of heat stress [41°C/1hr, (GD) 9] on serum levels (mean ± S.E) of glucose, calcium, osteocalcin and T_3 and T_4 hormones in rats.

Groups of rats	Glucose (mg/dl)	Calcium (mg/dl)	Osteocalcin (ng/ml)	Thyroxin (T3) (ng/ml)	Triiodothyronin (T4) (ng/ml)
Control (10)	98.471 ± 3.45	10.107 ± 0.13	0.730 ± 0.01	2.768 ± 0.18	7.138 ± 0.36
Heat stress (30)	119.789 ± 5.90*	8.515 ± 0.13*	0.790 ± 0.01*	1.426 ± 0.05*	5.790 ± 0.22*

* Significant ($P < 0.05$) compared with corresponding control one.

Table 3. Effects of maternal heat stress (41°C and 42 °C/1hr, GD 9) on the fetuses and placentae of rats on day 20 of gestation.

Treatment	Number of animals	Implantations (mean ±SD)	Resorptions (mean ±SD)	Alive (mean ±SD)	Fetal weight (mean ±SD)	Placenta weight (mean ±SD)	*Growth retardation (%) (−1SD)	(−2SD)
Sham	10	9.6 ± 2.3	1.0 ± 0	9.4 ± 2.0	34.23 ± 0.53	4.6 ± 0.6	8 (8.5%)	1 (1.1%)
41°C	14	8.6 ± 1.8	3.8 ± 3.0	6.7 ± 2.8**	22.1 ± 0.53***	4.9 ± 1.8	30(32.3%)**	36(38.7%)
42°C	10	7.6 ± 2.6	0.0 ± 0	7.4 ± 2.5	21.1 ± 0.66***	3.7 ± 0.9**	19(25.3%)	37(49.3%)**

* Percentages based on all living fetuses weighing 1SD or 2SD lesser than the control means.
** Significant ($P < 0.05$) compared with corresponding control one.
*** Highly Significant ($P < 0.001$) compared with corresponding control one.

Table 4. Incidence (%) of congenital malformations due to maternal heat stress (41°C and 41°C/1hr) on day 9 of gestation in rats.

Treatment	No. of embryos observed	Mandible/maxilla hypoplastic	Excencephaly with hemorrhage	Exopthalmia with cataract.	Low set microtia.	Facial clefts	Cleft palate	Short tail	Tongue protruding
Sham	30	10	0	0	3.3	0	0	6.6	0
41°C	44	15.9	13.6	2.2	6.8	9.0	2.2	9.0	9.0
42°C	30	30.0*	10.0	3.3	16.6	3.3	3.3	20.0	6.6

* Significant ($P < 0.05$) compared with corresponding control one.

Table 5. Skeletal abnormalities (%) due to maternal heat stress (41°C and 42 °C/1hr, GD 9) in rat fetuses of GD 20: Skull.

Bones	Control (%) n=30			41°C (%) n=32			42 °C (%) n=35		
	Normally ossified	Hypoplastic	Absent	Normally ossified	Hypoplastic	Absent	Normally ossified	Hypoplastic	Absent
Mandible	30 (100)	0	0	2(6.2)	27(84.3)	3(9.3)	7(20)	28(80)	0
Premaxilla	30 (100)	0	0	1(3.1)	28(87.5)	3(9.3)	6(20)	26(74.2)	3(8.5)
Maxilla	30 (100)	0	0	1(3.1)	31(96.8)	0	6(20)	27(77.1)	2(5.7)
Zygomatic	30 (100)	0	0	9(28.1)	22(68.7)	1(3.1)	7(23.3)	25(71.4)	2(5.7)
Nasal	30 (100)	0	0	5(15.6)	24(75)	3(9.3)	6(20)	26(74.2)	2)(5.7)
Frontal	30 (100)	0	0	6(18.7)	24(75)	2(6.2)	6(20)	26(74.2)	3(8.5)
Parietal	30 (100)	0	0	4(12.5)	26(81.2)	2(6.2)	5(16.6)	25(71.4)	5(14.2)
Inter parietal	30 (100)	0	0	6(18.7)	25(78.1)	1(3.1)	7(20)	26(74.2)	2(5.7)
Supra-occipital	30 (100)	0	0	8(25)	21(65.6)	3(9.3)	7(23.3)	24(68.5)	4(11.4)
Exoccipital	30 (100)	0	0	7(21.8)	23(71.8)	2(6.2)	4(13.3)	27(77.1)	3(8.5)
Temporal	30 (100)	0	0	4(12.5)	27(84.3)	1(3.1)	5(14.2)	27(77.1)	3(8.5)
Tympanic ring	30 (100)	0	0	5(15.6)	27(84.3)	0	4(13.3)	29(82.8)	2(5.7)
Hyoid	30 (100)	0	0	4(12.5)	27(84.3)	1(3.1)	2(6.6)	29(82.8)	4(11.4)
Ethmoid	30 (100)	0	0	5(15.6)	27(84.3)	0	2(6.6)	29(82.8)	5(14.2)
Presphenoid	30 (100)	0	0	4(12.5)	27(84.3)	0	4(13.3)	29(82.8)	2(6.6)
Basisphenoid	30 (100)	0	0	9(28.1)	23(71.8)	0	6(20)	29(82.8)	0
Basioccipital	30 (100)	0	0	9(28.1)	23(71.8)	0	5(16.6)	30(85.7)	0
Fontanelles	Normal	0	0	0	Wider(100)	0	0	Wider(100)	0

Table 6. Vertebral malformations (%) induced by maternal heat stress (41°C and 42 °C/1hr, GD 9) on fetuses of gestation day 20.

Region	Number	Control n = 30		41°C n=32		42 °C n=35	
		Arches	Bodies	Arches	Bodies	Arches	Bodies
	1-6	1(3.3)	1(3.3)	12(37.5)	2(6.2)	26(74.2)	9(25.7)
	7	29(96.6)	29(96.6)	20(62.5)	7(21.8)	9(25.7)	6(17.1)
	Hypoplastic	0	0	0	18(56.2)	0	22(62.8)
	Agenesis	0	0	0	5(15.6)	0	20(57.1)
Thoracic	12	1(3.3)	1(3.3)	3(9.3)	3(9.3)	6(17.1)	6(17.1)
	13	29(96)	29(96)	27(84.3)	27(84.3)	29(82.8)	29(82.8)
	14	0	0	0	0	0	0
Lumbar	1-5	0	0	2(6.2)	2(6.2)	3(8.5)	3(8.5)
	6	30(100)	30(100)	29(90.6)	29(90.6)	31(88.5)	31(88.5)
	7	0	0	1(3.1)	1(3.1)	1(2.8)	1(2.8)
Sacral	Agenesis	0	0	3(9.3)	5(15.6)	7(20)	12(34.2)
	1-6	29(96)	29(96)	27(84.3)	27(84.3)	26(74.2)	20(57.1)
	7	1(3.3)	1(3.3)	2(6.2)	0	2(7.5)	3(8.5)
Coccygeal	Agenesis	0	0	16(50)	4(12.5)	18(51.4)	16(45.7)
	1-4	18(60)	18(60)	15(46.8)	27(84.3)	16(45.7)	19(54.2)
	5-8	12(40)	12(40)	1(3.1)	1(3.1)	1(2.8)	0

Numerals in parentheses are percentages.

Table 7. Effect of maternal heat stress (41°C and 42°C/1hr, GD 9) on the development of ribs in rat fetuses of GD 20.

Ribs	Control (%) n=30		41°C (%) n=32		42 °C (%) n=35	
	Right	Left	Right	Left	Right	Left
13	30(100)	30(100)	28(87.5)	28(87.5)	27(77.1)	27(77.1)
10-12	0	0	4(12.5)	4(12.5)	8(22.8)	8(22.8)
Fusion	0	0	2(6.2)	2(6.2)	3(8.5)	3(8.5)
Hypoplasia	0	0	6(18.75)	6(18.75)	17(48.5)	17(48.5)

Table 8. Sternal malformation induced by maternal heat stress (41°C and 42°C/1hr, GD 9) in rat fetuses of GD 20.

Sternebrae	Control (%) n=30	41°C (%) n=32	42 °C (%) n=35
7	0	1(3.1)	2(5.7)
6	30(100)	13(40.6)	10(28.5)
4-5	0	17(53.1)	19(54.2)
Hypoplasia	0	10(31.2)	12(34.2)
Agenesis	0	1(3.1)	4(11.4)
Split	0	4(12.5)	6(17.1)

Table 9. Effect of maternal heat stress (41°C and 42 °C/1hr, GD 9) on limbs bones of rat fetuses of gestation day 20.

Bones	Control (%) n=30			41°C (%) n=32			42 °C (%) n=35		
	Normally ossified	Hypoplastic	Agenesis	Normally ossified	Hypoplastic	Agenesis	Normally ossified	Hypoplastic	Agenesis
Ilium	30 (100)	0	0	6(18.7)	26(81.2)	0	5(16.6)	30(85.7)	0
Ishchium	30 (100)	0	0	7(21.8)	25(78.1)	0	6(20)	29(82.8)	0
Pubis	30 (100)	0	0	8(25)	24(75)	0	8(26.6)	27(77.1)	0
Femur	30 (100)	0	0	10(31.2)	22(68.7)	0	9(30)	26(74.2)	0
Tibia	30 (100)	0	0	10(31.2)	22(68.7)	0	9(30)	26(74.2)	0
Fibula	30 (100)	0	0	5(15.6)	27(84.3)	0	5(16.6)	30(85.7)	0

Table 10. Effect of maternal heat stress (41°C and 42°C/1hr, GD 9) on the bones of the forepaw and hindpaw on day gestation 20.

Bones	Number	Control (%) n=30	41°C (%) n=32	42 °C (%) n=35
Metacarpals	Agenesis	0	2(6.2)	7(20)
	3	3(10)	7(21.8)	12(34.2)
	4	27(90)	23(71.8)	16(45.7)
Metatarsals	Agenesis	0	3(9.3)	5(14.2)
	3	0	4(12.5)	2(5.7)
	4	27(90)	25(78.1)	27(77.1)
	5	3(10)	0	1(2.8)
Tarsals	1	0	0	0
Phalanges (Forelimb)	Agenesis	0	27(84.3)	34(97.1)
	1-5	5(16.6)	5(15.6)	1(2.8)
	6-10	14(46.6)	1(3.1)	0
	11-12	11(36.6)	0	0
Phalanges (Hindlimb)	Agenesis	0	32(100)	35(100)
	1-5	8(26.6)	0	0
	6-10	22(73.3)	0	0

CHAPTER IV

DISCUSSION

DISCUSSION

1. Maternal Toxicity

In the present experiments maternal exposure to hyperthermia in a temperature controlled incubator was found to be very effective in raising the core body temperature. In many species including the rats there appears to be a threshold elevation capable of causing birth defects. This elevation is approximately 2.0-2.5°C above the normal temperature of that species. The threshold duration at a temperature elevation 2.0-2.5°C appears to be about 1hr (Germain et al., 1985; Kimmel et al., 1993a; Graham and Edwards, 1998). Maternal body weight gain showed reduction under heat stress because they consumed less food and water. In the present study, heat stressed mothers showed signs of sweating and exhaustion, and reduced physical activities. These signs and symptoms were very much similar to those reported by other investigators (Galina et al., 1982; Sugimoto et al., 1996; Cortes et al., 2000). Such effects could be interpreted as resulting from heat-induced skin vasodilation, increased ventilation rate and a decrease in ATP levels (Welch, 1992; Shapiro and Seidman, 1990; Gautier, 2000). The reduction in physical activity of the animals observed was possibly due to release of β-endorphin from the pituitary under heat stress condition (Galina et al., 1982).

Additionally a significant increase in serum level of glucose was observed in our heat stressed animals. A similar response was observed by Follenius et al., (1982) and Swolin-Eide and Ohlsson (1998) and explained as being a consequence of increased glucocorticoid secretion in pregnancy and in hyperthermic status. We did not estimate the glucocorticoids in these animals however; heat and other forms of stress are known to increase glucocorticoids secretion (Li et al., 2001). Glucocorticoids play a significant role in eliciting the adaptive mechanism of the organism adaptation against to stressful factors including hyperthermia by stimulating gluconeogenesis and because of their permissive actions on other hormones. In general, glucocorticoids, growth hormone, thyroid hormones and insulin-like growth factors have permissive action on development. In addition, high levels of glucocorticoids are believed to alter bone remodeling by decreasing bone formation and increasing bone resorption (Swolin-Eide and Ohlsson, 1998). Devarajan and Benz (2000) reported that glucocoriticoids decrease renal tubular calcium reabsorption leading to hypercalciuria. More chronically they interfere with intestinal calcium

absorption, alter vitamin D metabolism and lead to secondary hyperparathyroidism (Griffin and Ojeda, 1992). These findings agree with our results.

Many studies reported that hyperthermia caused significant decreases in serum thyroxin (T_4), triiodothyronine (T_3) (Horowitz and Meiri, 1985), thyroid stimulating hormone (TSH) (Tal and Sluman, 1975) in rats and reduction in plasma triiodothyronine (T_3) in cows (Baccari et al., 1983), chicken (Arjona et al., 1990; Yahav and Plavnik, 1999) and ewes (Bell et al., 1989). The results of these studies agree well with our findings in term of decreased levels of T_3 and T_4 in pregnant rats. Heat stress has been reported to lower the growth rates and thyroid function (Baccari et al., 1983). There was an inverse relationship between observed rectal temperature and growth rate. Maternal thyroid hormones are known to cross the placental barrier and influence fetal brain growth and development (Moore and Persaud, 1998) until such time when the fetus become self supporting by producing its own thyroid hormone. In the mouse embryos, the thyroid gland starts to function between embryonic day (ED) 15 to ED 17 (Kaufman and Bard, 1999). The fetal development in the rat is behind that of the mouse by about two days. Therefore, by deduction it can be assumed that the rat embryo starts producing thyroid hormones between ED 17 to ED 19. Maternal thyroid hormones during early pregnancy are essential for the development of fetal brain development until the fetal thyroid begins to function (Vulsman and Kok, 1996; Pop et al., 1999). They ensure a normal myelination by stimulating myelin gene (Rodriguez-Pena, 1999). These hormones are also important in craniofacial and eye development. Therefore, a series of defects might arise due to a loss of thyroid actions involved in anterior-posterior development of the head and face and the loss of thyroid dependent signals for cell differentiation, migration, and proliferation (Gamborino et al., 2001). Impairment of maternal thyroid hormone production is associated with severely impaired neurological development of the offspring (Pop et al., 1999) and abnormalities in structure and function of skeletal muscle (Janssen et al., 1978). This may explain the neural tube and skeletal defects that were observed in our studies. The present experiments showed a significant increase in serum osteocalcin level. Osteocalcin is a bone-specific protein. Its concentration in blood is reported to be a direct reflection of osteoblastic activity and bone formation (Gundberg et al., 1991). It also serves to promote an initial bone response to physiological stress before the normal hormonal regulations are elicited (Gundberg et al. 1991). Further more, a negative relationship between osteocalcin and total body calcium has also been reported (Delmas et al., 1983; Yasumure et al., 1987). This could explain the poor skeletal ossification that were observed in the present study.

II. Teratognic Outcome

Fetal teratogenicity can be manifested in (1) fetal death, (2) intrauterine growth retardation, or (3) malformations (Wilson, 1973). In hyperthermia studies exposure of mammalian embryos to hyperthermia to result in fetal malformations while more severe exposure results in embryonic or fetal death followed by resorption (Graham and Edwards, 1998).

1. Fetal death

The changes in the number of implantation were not significant in our experiments. This is understandable because, in the rat implantation occurs from GD 5 through GD 7 (Hebel and Stromberg, 1986). However, there were interlitter variations in embryonic resorption in response to maternal heat exposure.

2. Intrauterine growth retardation

The experimental groups showed a high incidence of growth retardation compared with the control group. In our experiments the amount of heat applied on animals was not enough to affect fetal life and placental weight but it was strong enough to induce intrauterine growth retardation. Growth retardation in our experiment was measured as an expression of the standard deviation (SD) of control mean fetal body weight. Those fetuses that weighed one or two SD less than the mean of the controls were regarded as growth retarded. Both 41°C and 42°C groups had a large number of fetuses at -2SD level but the 42°C group had a larger of -2SD fetuses. This means that growth retardation was a dose dependent response. Heat stress is known to causes disturbances of somite development, cell proliferation and differentiation, to denature enzymes, proteins and DNA fragmentation. Such changes might lead to cell death and finally inhibition of growth (Walsh et al., 1987; Breen et al., 1999; Germian et al., 1985; Rao et al., 1990; Germano et al., 1996; Mirkes et al., 1997; Edwards, 1972). A large number of the experimental fetuses were found to have their upper and lower jaw hypoplastic. The hypoplastic condition of the maxilla and mandible were possibly the reasons why protrusion of the tongue occurred. In control fetuses the mandibles contained a ramus and a body and they were connected at the symphysis menti. The remnant of the Meckel's cartilage was only found near the

symphysis menti. In contrast, a large number of the experimental fetuses were found to have the entire Meckel's cartilage persisting. The 42°C group showed a twofold increase in the incidence of persistent Meckel's cartilage compared to the 41°C group. Hyperthermia was observed to slow down the rate of the ossification process. This was evidenced by the presence of poorly ossified bones or only the presence of cartilage in some cases. There were two cases of exophthalmia associated with excencephaly; one in the 41°C and other on in the 42°C groups. The bones of the skull base and those of the orbit are hypoplastic and malformed in excencephaly fetuses. The small size of orbit is possibly the reason why the eye protrudes in such fetuses. The hyperthermic groups had a higher incidence of short tail than the controls which could be due to disturbance in somite development in hyperthermic groups. Such defects in the tail and eye were demonstrated by many studies (Breen et al., 1999; Edwards, 1968). Eye and brain defects were reported in hyperthermia studies reported by Arora et al., (1979) in vivo and by Mirkes (1985) in vitro. Variations in skeletal system have been reported in experimental animals due to heat stress by Cuff et al., (1993), Kimmel et al., (1993b) and Breen et al., (1999). Vertebral abnormalities were reported by Li et al., (1997) and Shiota (1988). Ours is possibly the first study that looked at the entire developing skeleton in hyperthermia experiments.

The control rat fetuses had well ossified bones of the skull that included the mandible, premaxilla, maxilla, zygomatic, nasal, frontal, parietal, interparietal, supra-occipital, exoccipital, temporal, tympanic ring, hyoid, ethmoid, presphenoid, basisphenoid and basioccipital bones. Fontanelles and suturel lines were appropriate for gestational age. But in contrast, in the experimental groups these bones were hypoplastic. The fontanelles and suturel lines were wide apart. This gives an indication of the slow process of development or delay in ossification. This effect was more extensive and severer in the higher temperature group. Furthermore, a higher incidence of hypoplasia of the ilium, ishchium and pubis and long bones of hindlimb was observed in the 42°C group than in the 41°C group. This is an indication of dose dependent fetal teratogenicity.

3. Malformations

The excencephalic embryos were found to have various other malformations of the craniofacial region and axial skeleton. Many of excencephaly fetuses had facial clefts. Fetuses with facial clefts, especially those with bilateral oblique facial clefts had cleft of the upper lip. Fetuses of the 41°C group had a higher incidence of facial clefts than those of the

42°C group. Furthermore, both experimental groups showed a low incidence of cleft palate. The early development of the neural plate occurs at the 8^{th} to 9^{th} day which becomes neural fold at the 9^{th} to 10^{th} day of gestation around which neural crest is formed (Adelmann, 1925; Baker et al., 1980). The pathogenic mechanism of neural tube defect may increase failure of the neural tube closure during the embryonic period (Von Recklinghausen, 1886) and/or reopening of the closed neural tube due to accumulation of cerbrospinal fluid (Morganis, 1769). One of the 42°C group fetuses had a skin appendage attached to the dorsal aspect of the right hindlimb. The mechanism of this anomaly is not known. The series of midline defects such as failure of neural tube closure, median facial clefs, cleft lip ect., indicate that hyperthermia affects the developmental of midline structures. The neural plate is neurectodermal in origin. The palatal shelves and the lip primordial are of bilateral origin. The neural crest is known to make a significant contribution to these structures. The results of our experiment indicate that hyperthermia preferentially affects the neural crest development.

The absence of skull vault was obvious in all exencephalic cases. The basicranial bones were hypoplastic. The basioccipital was more posteriorly placed than in normal fetuses. This position narrowed the foramen magnum. In anencephalics fetuses, absence of cranial vault exposes the brain tissue to the aberasive effects of the amniotic fluid. The damaged tissue tends to grow irregularly. This results in significant reduction in size and poor organization of the components of the basicranium. For example, the supraoccipital develops in two halves that subsequently fuse together. Non-fusion of the suproccipital centers possibly indicate retarded ossification. Fritz and Hess (1970), considered a dumb bell shaped supraoccipital as incomplete ossification. Ariyuki et al., (1980) provides a better scale for determining the extent of ossification of this bone which closely reflects the level of ossification of the skull as a whole. In the present study most of the experimental fetuses had only reached stage 2 of Ariyuki et al., (1980). Neural crest cells contribute to the bones of the face and most of the cranial vault. This is another evidence of neural crest involvement in hyperthermia-induced malformations in rat fetuses. The vertebral column and ribs are derived from paraxial mesoderm especially sclerotoms (Sadler, 2000). Further more disturbances in HOX gene expression due to heat stress at a critical period of development can induce axial skeletal malformations (Li et al., 1997).

The present study revealed the presence of 7 cervical, 13 thoracic and 6 lumbar vertebrae in the control. A large number of fetuses of heat stressed mothers had a fewer

number of ossified vertebrae. The higher the temperatures the higher was the decrease in cervical, thoracic, lumber, sacral and coccygeal arches and bodies. There was also an increased tendency to have a decreased number of sternebare with maternal heat elevation. Sternebare are of bilateral origin. The sternal primordial slowly comes close to each other and fuse to form the sternal bar. Heat stress causes failure of fusion of sternal primordia resulting in hemisternebare. Malalignment of the primordia results in malpositioning of the sternbrae. Reduction in number of ribs and sternebare indicate an early disturbance in somite formation. Smith et al., (1978), Kimmel et al., (1993b) and Breen et al., (1999) pointed to such skeletal anomalies in their heat stressed animal embryos.

III. Placenta

The placenta is the organ through which respiratory gases, nutrients, water and metabolic waste products are transported between the maternal and fetal systems. It is also involved in immune function, hormone production, and several metabolic processes and control of the fetal physiology, growth and development. Placenta in rats is considered hemochorial since the chorion is bathed in maternal blood. It is first represented by the visceral yolk sac alone until gestation day 11.5 at which point in time a structurally and functionally complex placenta is formed (Jollie, 1964; Davies and Glasser, 1968). Clinical studies on maternal hyperthermia have focused attention largely on the fetal outcome. Arora et al., (1979) found extensive thickening of the decidua basalis of their rat fetuses maternally exposed to heat from GD 6-10. In our study animals were exposed to heat stress for one hour on GD 9 to 41°C or 42°C. This treatment resulted in significant reduction in placental weight in addition to intrauterine fetal growth retardation and several malformations. The decidual thickening, hyalinization and lymphocytic infiltration observed in this study were similar to those reported by Arora et al., (1979). They related these changes to lasting inflammatory response of the maternal portion of the placenta to hyperthermia. Giant cell proliferation of the spongy zone was found to be a regular feature of all experimental placentas. These cells have two important functions, namely phagocytosis, and steroid hormone production. The areas where glycogen cells were found to be degenerated contained phagocytes. Polymormhonuclear leucocytes and platelet aggregations were also found in these sites. One of the most remarkable features of the hyperthermia group placentas was the reduction in number and degeneration of glycogen cells as judged by the Best's carmine test. Reduction of glycogen cells could have lead to

poor energy and impaired fetal growth as observed in this study. Light microscopic examination revealed altered labyrinthine architecture, poor vascularisation, perivascular fibrosis and persistence of fetal mesenchyme. All these changes pointed toward reduction in placental functional capacity. The EM revealed some more important details.

Layer III of the trichorial membrane had fewer plasma membrane infoldings (Fig.24) suggesting a reduction in its surface area and hence reduced placental function. These cells had less prominent pinocytic vesicles and glycogen in comparison to the controls. There were obvious pores in layer I through which layer II cytoplasm projected into the maternal sinusoid. The abundance of smooth endoplasmic reticulum and tubular mitochondria in the trophongiosal cells was suggestive of steroid hormone production. Remarkably they also stored lots of glycogen. This glycogen accumulation could be due to reduction its transport to the fetal compartment or a hightened glycogen metabolism within these cells. The glycogen containing cells also possessed an abundance of clear vacuoles, and smooth endoplasmic reticulum (sER). An interesting aspect of the morphology of such cells was the presence of electron dense material in a tubular network that was in the proximity of rough endoplasmic reticulum (rER). The nature and functions of this material was not determined in this study. The persistent fetal mesenchyme was found to have produced a lot of collagen and thus supporting our light microscopic observation of perivascular fibrosis. Heat–treated ewes were shown to have a greater placental concentration of protein containing hydroxyproline and glycine suggesting a greater collagen content. Our study has provided a mormphological basis for increased collagen in perivascular mesenchyme. The trophoblasts of the labyrinths also produced a fibrinoid substance which appeared to be secreted into the maternal sinusoid. Fibrinoid accumulation in the labyrinths together with the reduced placental weight in the experimental group, perivascular fibrosis, and the presence of cysts in the spongy zone might have contributed to a further reduction in placental function. Maternal heat has been shown to cause an increase in uterine blood flow, a reduction in placental weight, and a reduction in placental glucose transfer capacity without substantially changing umbilical blood flow, or fetal glucose uptake in the ewe (Bell et al., 1989; Thureen et al., 1992; Adrianakis and walker 1994). It is worth mentioning here that heat was applied to rats on GD 9, well before a functional placenta was in place and yet a multitude of microscopic and ultrastructural changes occurred that persisted to term. Heat shock has been shown to affect cell cycle and apoptosis leading to decreased number of cells (Gericke et al., 1989; Early et al., 1991). In heat shock, the transcriptional and translational mechanisms of the cell are affected by

preferential induction of heat shock proteins. Therefore Gericke et al., (1989) hypothesized that such phenomenon in embryos could lead to the absence of essential gene products at critical stages of development. Absence or excess of developmentally meaningful genes could result in abnormal development of both the embryo and the placenta. Abnormalities in placental structure and function could secondarily limit fetal growth as noted in the present study.

CONCLUSION

Heat stress (41°C and 42°C/1hr) on gestation day 9 was shown to be a potent teratogenic in pregnant rats. Results showed the adverse effects of heat on both maternal and fetal sides.

Pregnant rats whose rectal temperature reached about 2°C or more above normal body temperature showed signs of toxicity such as exhaustion, decreased water and food consumption, sweating, and diarrhea. In addition, significant increases in serum glucose and osteocalcin as well as decreases in serum calcium, T_3 and T_4 levels were observed.

Moreover, heat stress resulted in reductions in number of fetal implantations, number of live embryos, and fetal and placental weights. Heat stress also increased in fetal resorption rate, skeletal malformations, and growth retardation which could be due to alteration in embryo gene function. Furthermore, light and electron microscopic examination of placenta showed a series of degenerative changes in experimental group as compared with control.

CHAPTER V

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APPENDIX

APPENDIX

1. Gender's fluid:

Formaldehyde (40%)	5 ml
Alcoholic picric acid (1gm of picric acid in 100 ml of 95% ethanol) add 5ml of acetic acid before use	80 ml

2. Hematoxyline and eosin staining of placenta:

1. Dewax in xylene for 2 minutes each (two changes).
2. Dehydrate as following:
   - Keep 100 % ethanol for 1 min.
   - Keep 100% ethanol for 1min.
   - Transfer to 95% ethanol for 1 min.
   - Transfer to 70% ethanol for 1min.
   - Leave the slides in distilled water for 5 min.
3. Stain with Hematoxylin and Eosin for 4 min.
4. Leave in the running tap water for 5 min.
5. Transfer to acid ethanol.
6. Stain with Eosin for 30 sec.
7. Dehydrate as following:
   - Keep in 70% ethanol for 15 sec.
   - Keep in 95% ethanol for 15 sec.
   - Transfer to 100% ethanol for 1 min.
   - Leave in 100% ethanol for 1 min.
8. Clear with two changes of xylene, 2 min. for each.
9. Mounting.

3. Best Carmine test:

1. Deparaffinize for 3 minutes each. (two change)
2. Transfer in 100% ethanol for 3 minutes each. (two change)
3. Dip 2 times in 70% alcohol.
4. Dip 15 times in distilled water.
5. Stain in hematoxylin dye for 5 minutes.
6. Wash in running tap water for 5 minutes.
7. Keep in best carmine working solution for 30 minutes.
8. Treat with differentiating fluid for 5 minutes.
9. Rinse quickly in 80% alcohol.
10. Dehydrate, clear, and mount.

4. Working solution:

Carmine stock solution	30.0 ml
Ammonium hydroxide	25.0 ml
Methyl alcohol	25.0 ml

5. Differentiating fluid:

Absolute ethyl alcohol	16.0 ml
Methyl alcohol	8.50 ml
Distilled water	20.0 ml

ARABIC SUMMARY

الملخص

تظهر بعض التشوهات التشريحية للمواليد عند الميلاد. أسباب هذه العيوب تكمن في عوامل وراثية و بيئية عديدة. الغرض من هذه الدراسة هو تحديد تأثير الإجهاد الحراري لأمهات الفئران الحوامل على تطور الجهاز الهيكلي للأجنة. استخدمت في هذه الدراسة ٧٤ فارة أنثى من نوع وستر عند اليوم التاسع من الحمل، جلبت من بيت الحيوان التابع لكلية الطب. قسمت الفئران في هذه الدراسة إلى مجموعتين: المجموعة الأولى تحتوى على أربعين فارة ،ثلاثون منها عرضت ل ٤١ درجة منوية وعشرة عند حوالي ٢٥ درجة منوية (العينة الضابطة) لمدة ٦٠ دقيقة. سحب الدم مباشرة من الحيوانات و فصل مصل الدم وتم حفظة عند ٨٠ درجة منوية تحت الصفر لحين استخدامه.

قدرت مستويات هرموني الثيروكسين و تراى أيودوثيرونين في مصل الدم بالإضافة إلى مستويات الجلوكوز والكالسيوم و الاوستى كالسين (بروتين العظام). و بمقارنة نتائج الفحص مع العينة الضابطة وجد أن هناك انخفاض معنوي في معدلات هرموني الثيروكسين و تراى أيودوثيرونين والكالسيوم. بالإضافة إلى الزيادة المعنوية في مستويات الجلوكوز و الاوستى كالسين في الحيوانات المجهدة حراريا.

أما المجموعة الثانية اشتملت على ٣٤ فارة، ١٤ منها عرضت للإجهاد الحراري عند ٤١ درجة منوية، وعشرة عند ٤٢ درجة منوية و عشرة و ضعت عند ٢٥ درجة منوية (العينة الضابطة) لفحص الشكل المورفولوجى و الهيكل العظمي لأجنتها. و بمقارنة النتائج مع العينة الضابطة وجد هناك انخفاض في عدد الأجنة المنغرسة و عدد الأجنة الحية ووزن المشيمة بالإضافة إلى التشوهات في الشكل المورفولوجي و الهيكل العظمى (عظام الجمجمة، العمود الفقري، الأضلاع، السلاميات...الخ) مما يدل على تأثر عمليات تشكل العظم بالحرارة. وكان الهدف من هذه الدراسة هو ايضاح تأثير الإجهاد الحراري على أجنة هذه الفئران وبيان درجة شدة التشوهات العظمية اعتمادا على درجة الحرارة.

كذلك جمعت عينات المشيمة من المجموعات المعاملة لفحصها تحت المجهر الضوئي و الإلكتروني. و دل الفحص على أن المشيمة في الحيوانات المعاملة بالإجهاد الحراري تحتوى على الكثير من المتغيرات مقارنة بالعينة الضابطة.

جامعة الإمارات العربية المتحدة
عمادة الدراسات العليا
برنامج ماجستير البيئة

تأثير الإجهاد الحراري على نمو العظام في الجنين

رسالة مقدمة من الطالبة
نورة مساعد عبد الله المساعد المنهالي
بكالوريوس في العلوم تخصص علوم الحياة
كلية العلوم جامعة الإمارات العربية المتحدة
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استكمالاً لمتطلبات الحصول على درجة الماجستير في
(علوم البيئة)

جامعة الإمارات العربية المتحدة

مايو – ٢٠٠٢