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Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo

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Abstract 1. Tissue-specific profiles of the expression of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) and the concentrations of reduced glutathione (GSH) during the development of the chick embryo were investigated.

2. The liver, brain, yolk sac membrane (YSM), kidney, lung, heart and skeletal muscles were collected at the following days of embryo development: 10, 11, 13, 15, 17, 19, 21 and 22 (day-old chicks).

3. The different tissues of the embryo displayed distinct development strategies with regard to the acquisition of antioxidant capacity. In the liver the specific activity of SOD increased between days 10 and 11 of development, then significantly decreased up to day 15 and remained at the same value during the rest of the developmental period. GSH-Px specific activity increased through the time of development. CAT had 2 peaks of specific activity at day 10 of the development and in day-old chicks.

4. The brain was characterised by comparatively high SOD-specific activity especially during the last days of incubation. The specific activities of GSH-Px and CAT were low throughout development.

5. In the YSM maximal GSH-Px and CAT-specific activities were found on day 15 of incubation. In the kidney and heart GSH-Px-specific activity increased at hatching time. CAT-specific activity in the kidney increased just after hatching.

6. It is concluded that each tissue studied expressed a profile of antioxidant defence mechanisms to deal with oxidative stress at hatching time.

INTRODUCTION

The development of the avian embryo is dependent on aerobic metabolism and, in particular, features the β-oxidation of fatty acids derived from yolk lipids (Stock and Metcalfe, 1987; Noble and Cocchi, 1990). The rate of oxygen consumption increases dramatically from about the mid-period of the 21-day development, partly because of the growth of the embryo but tends to become relatively constant during the last few days before hatch (Freeman and Vince, 1974; Vleck and Hoyt, 1991). Such increases in mitochondrial respiration and oxygen uptake are obligatory aspects of embryonic development, providing the energy for tissue growth, transport of nutrients from the yolk, maintenance of the heartbeat and other essential functions. However, it is likely that these beneficial aspects may be accompanied by potentially harmful effects because high rates of energy metabolism can lead to the production of reactive oxygen species and other free radicals which can cause damage to cellular macromolecules (Halliwell, 1994). In particular, polyunsaturated fatty acids are very vulnerable to free radical-induced peroxidative damage (Porter *et al.*, 1995) and it is pertinent that the lipids of several tissues of the chick embryo are highly unsaturated (Noble and Cocchi, 1990; Maldjian *et al.*, 1996).

In the chick embryo, protection against peroxidative damage is provided by the concerted action of a range of antioxidant components (Surai *et al.*, 1996). For example, vitamin E, the major lipid-soluble antioxidant which breaks the chain reaction of lipid peroxidation, is transported from the yolk to the embryonic tissues during development (Noble *et al.*, 1993; Gaal *et al.*, 1995; Surai *et al.*, 1996). Moreover, ascorbic acid, a major water-soluble antioxidant, is synthesised in the yolk sac membrane and transported to various tissues, particularly to the brain (Wilson, 1990; Surai *et al.*, 1996). However, the first line of defence against reactive oxygen species consists of a trio of antioxidant enzymes. Superoxide dismutase (SOD) reacts with the superoxide radical, formed as a result of electron leakage from the electron transport chain, converting it to hydrogen peroxidase (Jaeschke, 1995).Both catalase (CAT) and glutathione peroxidase (GSH-Px) are able to convert hydrogen peroxide to water (Fantel, 1996). Thus these 3 enzymes act in concert. GSH-Px also detoxifies lipid hydroperoxides (Yu, 1994) and thus acts synergistically with vitamin E to attenuate the peroxidative cycle (MacPherson, 1994).

An imbalance between the rate of free radical generation and antioxidant capacity in the embryo could potentially result in permanent damage to

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tissues at a sensitive period of their development. For example, encephalomalacia in chicks is associated with inadequate concentrations of vitamin E in the cerebellum (Dror *et al.*, 1976, Fuhrmann and Sallman, 1995). The hatching and immediate post-hatching periods are associated with dramatic increases in the rates of energy metabolism as well as with exposure to atmospheric oxygen (Freeman and Vince, 1974). Thus, it is suggested that the acquisition of antioxidant capacity in the embryo may not only serve to protect the embryonic tissues but may also represent a preparation for the potential oxidative hazards associated with hatchability.

Antioxidant enzymes have been shown to have different development profiles in the chick embryo liver and brain (Gaal *et al.*, 1995) and it has been suggested that changes in SOD and GSH-Px which occur in chick brain during the final 2 weeks of embryonic development are associated with the timing of neuronal and glial proliferation and differentiation (Wilson *et al.*, 1992).

The aim of the present study was to determine tissue-specific profiles of the expression of antioxidant enzymes SOD, GSH-Px and CAT and the concentrations of reduced glutathione (GSH) during the development of the chicken embryo.

MATERIALS AND METHODS

Eggs and embryos

Fertilised eggs from broiler-breeder hens (Ross 1 strain) were obtained from a commercial hatchery and incubated at 37·8°C and 60% relative humidity in a forced-draught incubator with automatic egg turning. The chickens hatched after 21 d of incubation and were maintained in the incubator for 1 d with access to drinking water but with no food provision (this day is referred as 22nd day of development). At each developmental stage 5 embryos or chicks were used. Brain, liver, lung, kidney, heart, thigh muscle and yolk sac membrane (YSM) were dissected, weighted and tissue samples were immediately prepared for analysis of antioxidant enzyme activities and GSH as described below.

Assay of antioxidant enzyme activities

Tissue samples were washed in potassium phosphate buffer (10 mM, pH 7·4) at 4°C and then homogenised in 9 volumes of the same buffer, supplemented with 30 mM potassium chloride, as described by Wilson *et al.* (1992). The protein content was determined in an aliquot of the homogenate using a Bio-Rad dye-binding kit system (Bio-Rad Laboratories, Muchen, Germany). The remainder of the homogenate was centrifuged (3500 *g*, for 30 min at 4°C) and the enzyme activities in the resulting supernatant were determined. GSH-Px and SOD were determined spectrophtometerically using kit systems supplied by Randox Ltd. (Crumlin, Nothern Ireland). The GSH-Px assay is based on the enzyme-catalysed oxidation of GSH by cumene hydroperoxide coupled to the reduction of the oxidised GSH by NADPH. Units of GSH-Px activity are expressed as μmol NADPH oxidised/min. The SOD assay employs xanthine and xanthine oxidase to generate superoxide radicals which are detected by formazan dye formation. Units of SOD activity are defined by the amount of the enzyme required to inhibit the rate of formazan dye formation by 50%, under the conditions specified by the kit manufacturer. CAT activity was measured by the method of Aebi (1984), based on the decomposition of hydrogen peroxide. Units of CAT activity are defined as μmol hydrogen peroxide decomposed/min. The activities of the 3 enzymes are expressed as units/mg homogenate protein.

Reduced GSH was determined by the method of Griffith (1980) involving the determination of total GSH with an enzymatic recycling assay with GSH reductase; oxidized GSH was determined in the presence of 2-vinylpyridine and GSH was calculated by difference.

Statistical analysis

Results are presented as mean ± SE of measurements on tissue from 5 replicate embryos at each developmental stage. Statistical analysis was performed by 1-way ANOVA and Students *t*-test.

RESULTS

The specific activity of GSH-Px in the embryonic liver increases continuously during the 2nd half of the *in vivo* developmental period so that the activity at hatching was 3·0 times greater ($P<0·001$ than that at day 10. The most rapid increase occurred between days 11 and 15 with a much more gradual increase thereafter. The expression of GSH-Px in the YSM also increased (by 1·8 times) between days 11 and 15 ($P<0·001$) but decreased thereafter. By contrast, the specific activity of GSH-Px in the brain was very low and relatively constant through the 2nd half of embryonic development. Thus, by the time of hatching, the specific activity of the enzyme in the liver was 6·1 times greater than that in the brain (Figure 1).

GSH-Px activities in the embryonic kidney, lung, heart and skeletal muscle were measured between day 15 of embryonic development and 1 day after hatching (Figure 2). During this period, the enzyme specific activity in the kidney increased gradually by 1·6 fold ($P<0·01$); the amount in the heart remained approximately constant; the specific activity in skeletal muscle decreased gradually by 30% ($P<0·02$); the specific activity in lung decreased by 27% ($P<0·01$) between days 15 and 19 then increased by the same amount ($P<0·01$) by day 1 after hatching.

The developmental expression of SOD in the

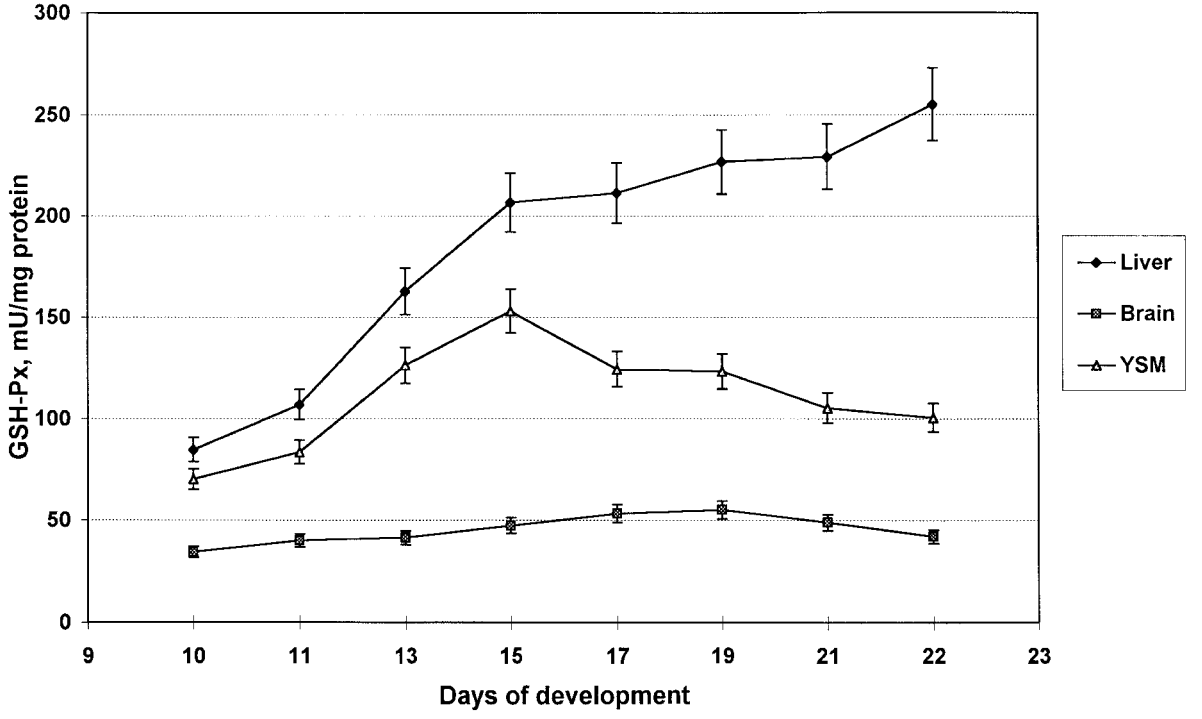

Figure 1. *Glutathione peroxidase activity in chick embryo liver (♦), brain (□) and YSM (∇). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

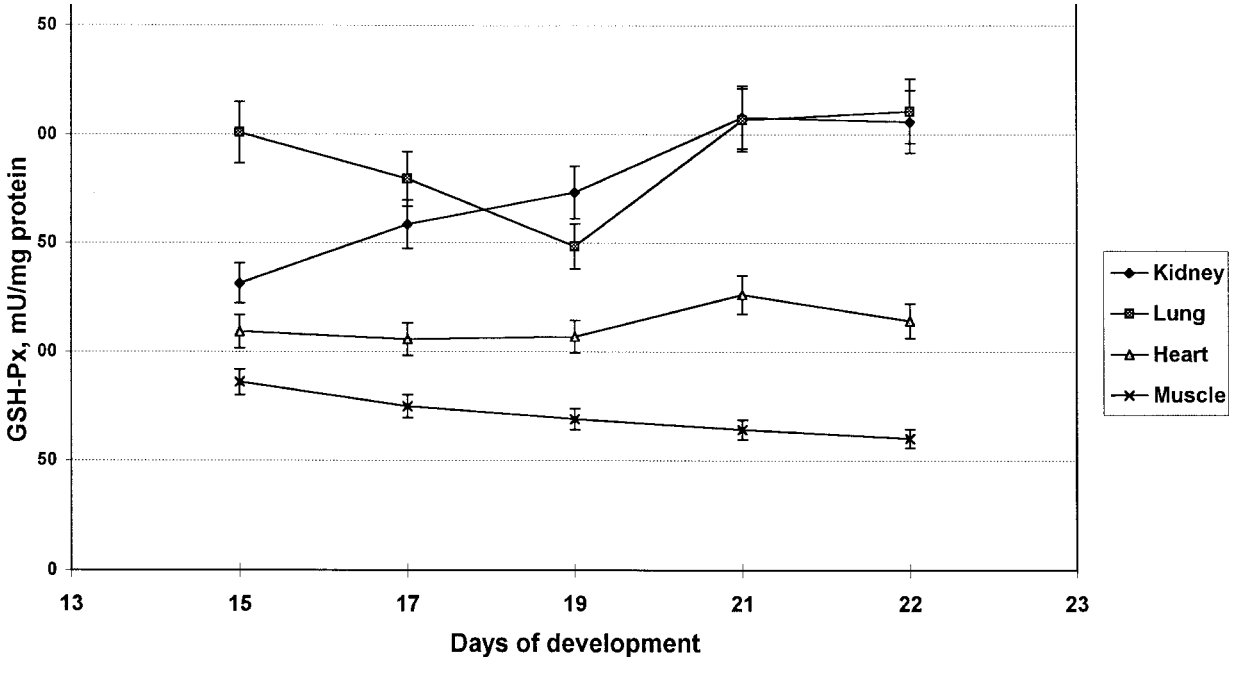

Figure 2. *Glutathione peroxidase activity in embryo tissues (♦-kidney, □-lung, ∇-heart and ×-skeletal muscle). Values are means of measurment of 5 replicate tissue samples with SE indicated by the error bars.*

various tissues (Figure 3 and 4) followed a pattern that was totally different from that of GSH-Px. In the liver, SOD specific activity was maximal at day 11 but decreased sharply by day 15 ($P<0{\cdot}001$) and remained relatively constant thereafter. By contrast the specific activity of SOD in the brain from day 15 onwards was approximately 2 times higher than in the liver ($P<0{\cdot}01$). In the YSM SOD specific activity increased gradually between days 10 and 15 and then decreased gradually between day 15 and hatching. The specific activities of SOD in kidney, lung, heart and skeletal muscle all showed a gradual decrease between day 15 and hatching.

The profile of CAT expression (Figures 5 and 6) in the embryonic tissues differed from those of both GSH-Px and SOD. The specific activity of CAT in the liver was high at day 10, decreased by 50% ($P<0{\cdot}001$) by day 19, then increased slowly by 1·9-fold ($P<0{\cdot}001$) over the hatching period. By contrast, only very low specific activities of CAT were detected in the brain throughout the period studied. Thus, at hatching, the liver had 18·8 times

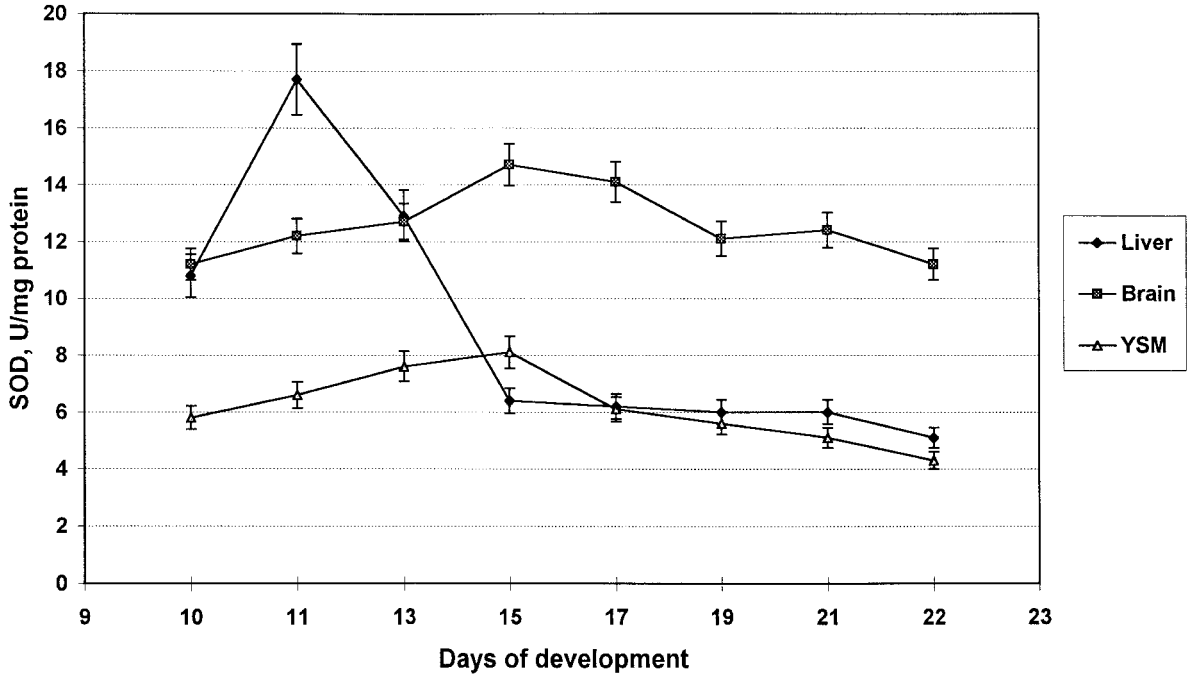

Figure 3. *Superoxide dismutase activity in chick embryo liver (♦), brain (□) and YSM (∇). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

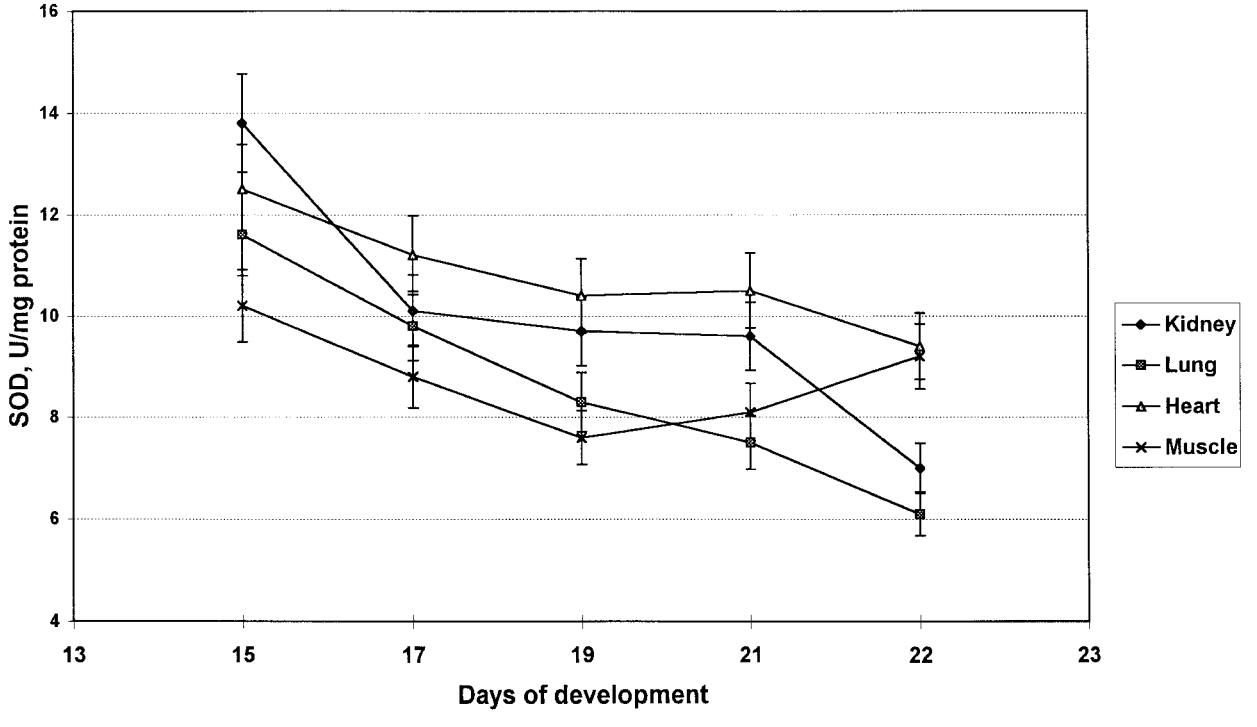

Figure 4. *Superoxide dismutase activity in embryo tissues (♦-kidney, □-lung, ∇-heart and ×-skeletal muscle). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

more CAT activity than the brain. Significant levels of CAT activity were present in the YSM with the maximal activity at day 15.

The pattern of CAT expression in kidney was similar to that in liver, again with a dramatic, 2-fold ($P<0{\cdot}001$) increase over the hatching period. The specific activities of CAT in lung, heart and skeletal muscle were much lower and relatively constant during development.

Changes in the tissue concentration of reduced GSH are shown in Figures 7 and 8. In the liver, brain and YSM, the greatest amounts of GSH were observed at day 10, with lower amounts after that time. Concentrations of GSH in kidney, lung, heart and skeletal muscle were relatively constant between day 15 and hatching.

DISCUSSION

Tissue-specific antioxidant protection

At hatching, tissue phospholipids contain large amounts of highly polyunsturated fatty acids,

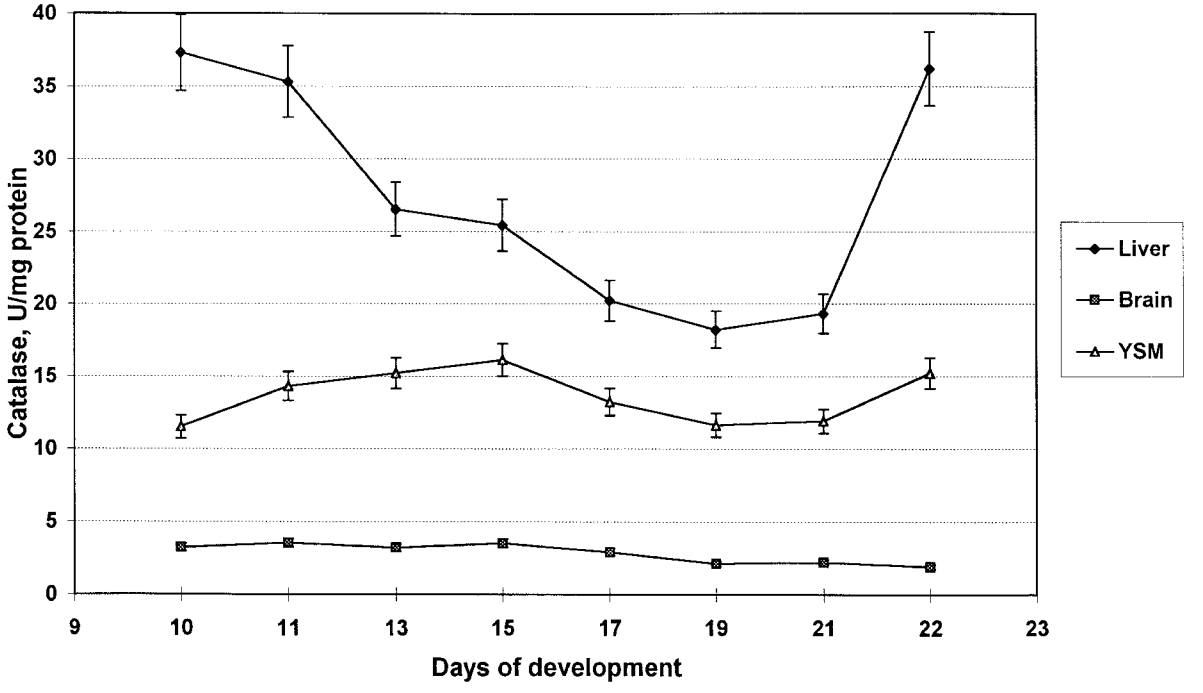

Figure 5. *Catalase activity in chick embryo liver (♦), brain (□) and YSM (▽). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

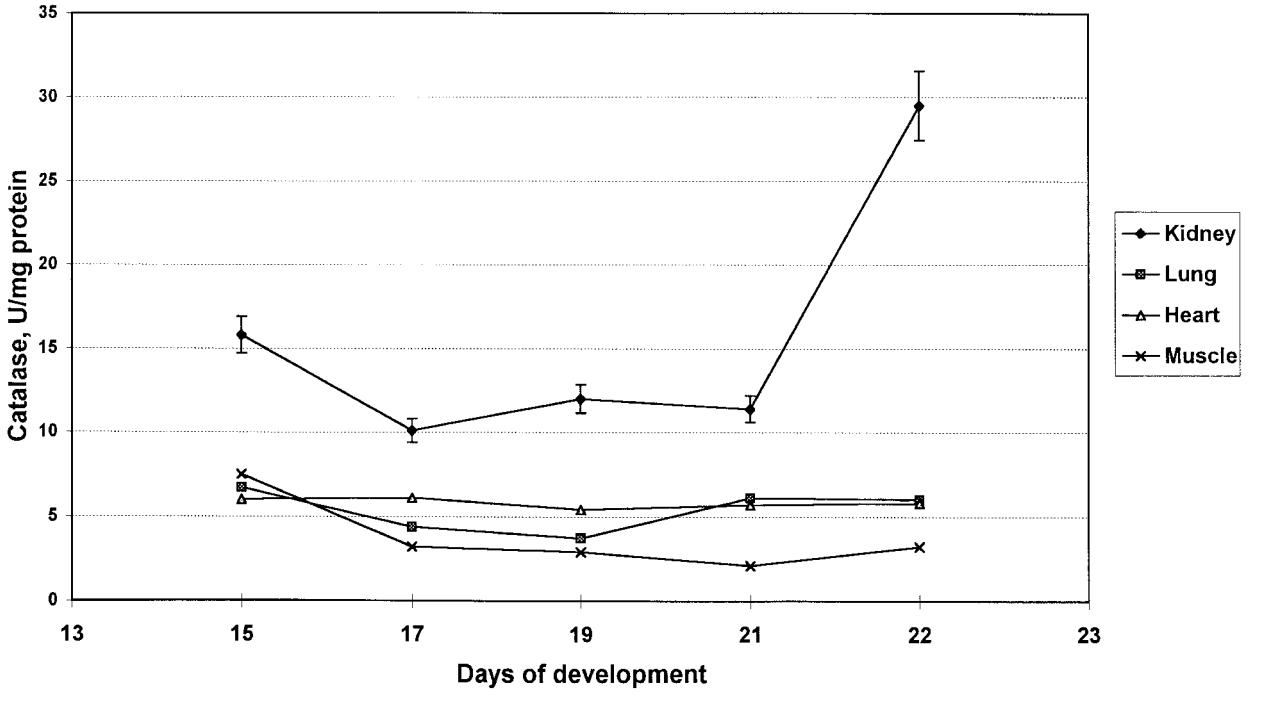

Figure 6. *Catalase activity in embryo tissues (♦-kidney, □-lung, ▽-heart and ×-skeletal muscle). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

particularly arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acid (Noble and Speake, 1997) and require a considerable degree of antioxidant defence against peroxidation.

The specific activities of the 3 antioxidant enzymes in the liver changed differently during development. SOD activity increased between days 10 and 11 of development, then decreased significantly in specific activity up to day 15 and remained with the same activity during the rest of the developmental period. Similarly there was no difference in SOD specific activity in the embryonic liver between days 12 and 18 (Wilson *et al.*, 1992). GSH-Px specific activity in the embryo liver increased through the time of development: from 84·7 mU/mg protein on day 10 to 254·9 mU/mg protein on day 22, in agreement with Wilson *et al.* (1992) and Gaal *et al.* (1995).

Comparison of the specific activities of GSH-Px in various tissues showed that the greatest activity was found in the embryo liver at all stages. In general, changes in GSH-Px specific activity patterns during development were very similar to those found for vitamin E, carotenoids (Surai *et al.*,

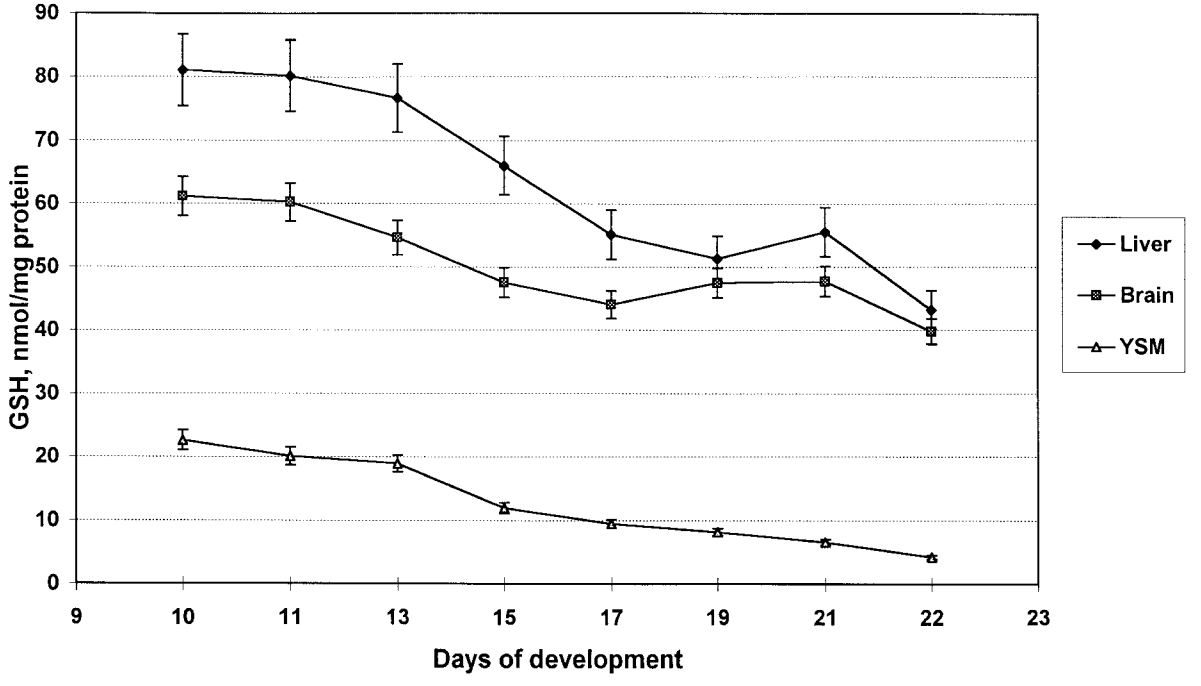

Figure 7. *Reduced glutathione concentration in chick embryo liver (♦), brain (□) and YSM (∇). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

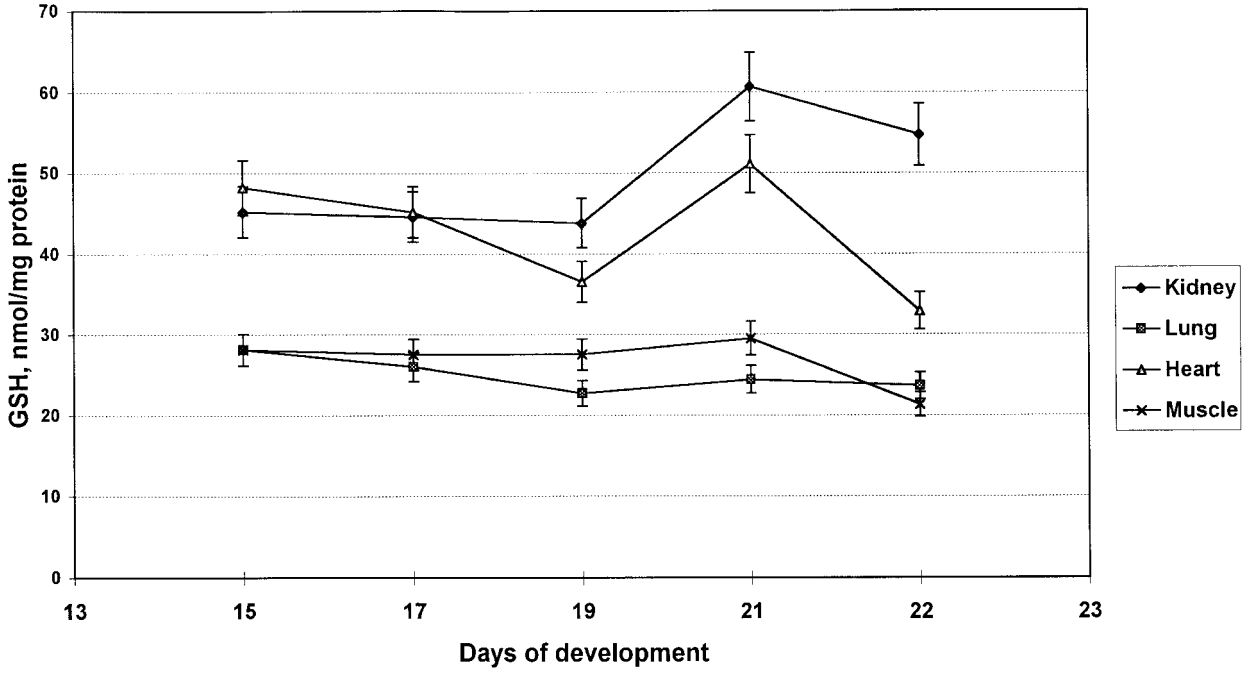

Figure 8. *Reduced glutathione concentration in embryo tissues (♦-kidney, □-lung, ∇-heart and ×-skeletal muscle). Values are means of measurement of 5 replicate tissue samples with SE indicated by the error bars.*

1996) and vitamin A (Gaal *et al.*, 1995). In the mouse embryo, the liver was the organ with the greatest amounts of GSH-Px mRNA (Dehaan *et al.*, 1994) GSH-Px probably plays a primary role in antioxidant defence of the liver, effectively removing hydrogen peroxide and lipid hydroperoxides from cells whereas CAT may be less important. Nevertheless, the concentration of GSH, a primary cell water-soluble antioxidant, gradually decreases throughout development. GSH is considered to be critical in protection against embryotoxicans (Tiboni *et al.*, 1997). CAT had 2 peaks of specific activity, at day 10 of embryonic development and in day-old chicks. After hatching the liver displayed CAT activity similar to that in the kidney but much higher than in the other tissues.

Thus, an accumulation of natural antioxidants vitamin A, E and carotenoids, a comparatively high vitamin C concentration and an increased GSH-Px specific activity correspond with the extensive metabolic and detoxifying function of this organ and together with comparatively high concentration of iron (Richards, 1997), which is associated with free radical formation protect liver lipids against peroxidation (Surai *et al.*, 1996).

The present data indicate that SOD is the main

enzyme of antioxidant defence in the brain, especially during the last days of incubation when SOD activity in brain was significantly greater than in other tissues. However, GSH-Px and CAT specific activities in the brain were low and it is unlikely that they are the primary defence in this tissue. GSH concentration also decreased with development. In contrast, in mammals the quantity of GSH in the foetal brain remained comparatively stable (Dreosti and Partick, 1987).

Endogenous concentrations of vitamin E in the embryonic brain are extremely low, carotenoids are not detectable and the ascorbic acid concentration is very high (Surai *et al.*, 1996). It was suggested that effective recycling of vitamin E by ascorbic acid is the main protective mechanism in the brain *in vivo* but is insufficient in providing protection to the brain in stress conditions during *in vitro* incubation (Surai *et al.*, 1996). From the present results it is clear that SOD may be another important protective element in the embryonic brain.

The antioxidant system of the brain is of great importance because of the development of nutritional encephalomalacia described in young chicks (Peppenheimer and Goettsch, 1931; Marthedal, 1973) as a result of vitamin E deficiency enhanced by unsaturated fatty acids (Dror *et al.*, 1976). The disease is associated with peroxidative dysfunction (Fuhramnn and Sallmann, 1995) and is characterised by localised haemorrhage and necrosis in the cerebellum, leading to ataxia, prostration and death (Hassan *et al.*, 1990). Nevertheless, the precise mechanisms of the development of the disorder have remained unclear.

In the YSM, maximum activities of the antioxidant enzymes were found on day 15 of development. GSH concentrations in the YSM gradually decreased throughout the development in such a way that at hatching time the concentration was 5 to 9 times lower compared to those in other tissues studied. During the 2nd half of incubation, YSM contains comparatively high concentrations of vitamin E, carotenoids (Surai *et al.*, 1996) and vitamin A (Gaal *et al.*, 1995) but low concentrations of ascorbic acid (Surai *et al.*, 1996). During this time the susceptibility to peroxidation decreased (Surai *et al.*, 1996). It is probable that increased activities of GSH-Px and CAT at day 15 and a sharp increase in peroxide accumulation at day 16 of development (Surai *et al.*, 1996) indicate oxidative stress at this stage. It seems that at the end of the incubation when the YSM involution begins, the protective mechanisms in this organ decreased as well.

In the lung GSH-Px activity decreased on day 19 and again increased just before hatching but GSH concentrations remained the same during development. Day 19 is a time when the animal is changing from respiration through eggshell pores, by way of the chorioallantoic membrane, to respiration through the lung and in such conditions an increased GHS-Px activity would be of great importance.

In general, comparatively low specific activity of SOD, moderate amounts of vitamin E and carotenoids (Surai *et al.*, 1996) together with a high iron concentration (Richards *et al.*, 1991) and exposure to the oxygen at hatching time place the lung at risk from possible free radical formation, lipid peroxidation and tissue injury. In postnatal chick development an impaired antioxidant system of the lung is an important part in the development of Pulmonary Hypertension Syndrome (PHS) (Bottje and Wideman, 1995) and attenuation of PHS mortality was achieved by vitamin E implanting to birds (Bottje *et al.*, 1995).

In the heart the GSH-Px specific activity was half that of the liver or lung and comparatively stable during embryo development with a slight increase before hatching. The embryonic lung was characterised by a high SOD specific activity. In the heart the specific activity of CAT was low and did not change during development. The embryonic heart is characterised by moderate amounts of vitamin E and low concentrations of carotenoids (Surai *et al.*, 1996). High proportions of arachidonic and docosahexaenoic acids in the heart phospholipids are associated with moderate antioxidant protection and suggest that, in stress conditions, the heart will be vulnerable to oxidative damage. For example, the embryonic heart was characterised by comparatively high susceptibility to lead-induced lipid peroxidation *in vivo* (Somashekaraiah *et al.*, 1992).

In the kidney GSH-Px specific activity increased during development reaching its maximum at hatching time. On the other hand, the specific activity of SOD decreased significantly during development. Nevertheless, it was comparatively high compared with other tissues. During development CAT specific activity was comparatively high and stable with a significant increase at hatching time.

Vitamin E accumulation in the embryonic kidney followed the same pattern as in the liver (Surai *et al.*, 1996). In the kidney the ascorbic acid concentrations were comparatively high and did not change during development up to the time of hatching (Surai *et al.*, 1996). Thus the antioxidant system of the embryonic kidney includes vitamins A, E and C and comparatively high activities of antioxidant enzymes and probably has an adequate protective potential at hatching time to deal with potentially harmful metabolic end products as a result of their functional activity.

In the thigh muscle the specific activities of the antioxidant enzymes varied little during development. Skeletal muscle is characterised by a comparatively low and stable vitamin E concentration. In contrast, the ascorbic acid concentration decreased significantly during development (Surai *et al.*, 1996). Thus, the antioxidant system of the muscle is not very potent and imbalance of the natural

antioxidants in postnatal development may cause such diseases as muscle degeneration (Machlin and Shalkop, 1956).

CONCLUSIONS

Antioxidant enzymes are a major cell defence against acute oxygen toxicity (Harris, 1992), are responsible for the detoxification of reactive oxygen species and preventing lipid peroxidation (Gille and Sigler, 1995). The expression of their activities is regulated in tissue-specific fashion at the gene level (Bermano *et al.*, 1995; Tsan, 1997; Weiss *et al.*, 1997). They are considered as important embryoprotective enzymes (Ozolins *et al.*, 1996; Fantel, 1996) during organogenesis when increased exposure to oxidant-derived free radicals or inadequate systems for antioxidant defence could alter cellular response at critical points in development (Fantel, 1996; Allen and Venkatraj, 1992).

The results of the present study indicate that different tissues of the embryo display distinct development strategies with regard to the acquisition of antioxidant capacity. A low oxygen pressure in embryos during development seems to have been retained in the course of evolution to protect the vulnerable developing tissues from the damage caused by the action of reactive oxygen species (Ar and Mover, 1994) as far as the rate of free radical generation in cells is influenced by ambient oxygen concentration (Turrens *et al.*, 1982). The reactivity of oxygen radicals and their derivatives implicate them as possible effectors of oxygen-mediated changes in gene expression (Allen and Venkatraj, 1992).

During chick embryo development there is an antioxidant/pro-oxidant balance in the tissues which is responsible for normal embryonic development and posthatch chick viability. It is suggested that an accumulation of natural antioxidants, vitamins A, E and carotenoids, as well as an increase in GSH-Px activity in the embryonic liver may have adaptive significance and were developed during evolution to protect unsaturated lipids against peroxidation during hatching stress conditions. On the other hand, an increased SOD activity and very high concentrations of ascorbic acid in the brain may be considered as another tissue-specific adaptive mechanism in embryo development. In general, it is necessary to point out that during mammalian prenatal development the antioxidant system is considered as immature (Allen and Venkatraj, 1992; Fantel, 1996) with maturation occurring postnatally. Probably the same is true in avian embryo development. An embryo mostly relies on natural antioxidants accumulated in the egg yolk as a result of their transfer from the maternal diet and after hatching antioxidant enzymes will play a more important role in tissue protection against lipid peroxidation. It is especially important because a reduced antioxidant activity has been reported in tissues of young chickens which suffered from encephalomalacia, exudative diathesis and muscular degeneration (Machlin and Gordon, 1962).

Our previous data indicate that there is a high correlation between vitamin E concentrations in the maternal diet and egg yolk (Surai *et al.*, 1995) and respectively, in embryonic tissues (Surai *et al.*, 1997*a*) and increased vitamin E concentrations in the embryonic liver and brain result in decreased tissue susceptibility to lipid peroxidation (Surai *et al.*, 1997*b*). Although, as yet, there is little understanding of the regulation of antioxidant systems in avian embryo development, a nutritional way to increase their efficiency seems to be promising.

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