Specific limb abnormalities induced by hydrogen peroxide in tadpoles of Indian jumping frog, *Polypedates maculatus*

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Hydrogen peroxide (H₂O₂), one of the reactive oxygen intermediates (ROI) and a potential inducer of nuclear transcription factors induces consistent type of abnormal limb development (truncated with bent skeletal elements) in the tadpoles of Indian jumping frog, *Polypedates maculatus*.

One of the intriguing riddles of the developmental biology is to unveil the molecular switches that regulate cell differentiation. In this connection, hydrogen peroxide, a central oxygen metabolic intermediate has recently gained much attention as a possible signaling molecule involved in signaling transduction pathways. In addition, it has been reported that hydrogen peroxide and its precursor, superoxide radicals can stimulate growth and growth responses in many cell types when added exogenously. However, there is no report available on the effect of hydrogen peroxide on development of animals in general and tadpoles of amphibians in particular. It is well established that hydrogen peroxide, one of the reactive oxygen intermediates (ROI) induces activation of many transcription factors including the nuclear transcription factor Kappa B (NF-kB) in Hela, endothelial cells, several T cell line and some primary cells. Hydrogen peroxide also induces expression of proteins encoded by NF-kB regulated genes. In the present study the effect of hydrogen peroxide has been investigated on limb development and tail regeneration in amphibian system taking it as a model to understand the role of hydrogen peroxide during development and differentiation.

**Materials and Methods**

Three types of hydrogen peroxides, *i.e.* hydrogen peroxide (H₂O₂), cumene hydrogen peroxide (cum-H₂O₂) and tertiary butyl hydrogen peroxide (Tert-H₂O₂) were used. Egg nests of *Polypedates maculatus* (Anura : Rhacophoridae) were collected from nature during the monsoon period of the year 1998-2000 and tadpoles were reared in the laboratory following standard procedure. Tadpoles of three specific stages namely pre limb bud, limb bud and limb paddle stage (Gosner Stage 25, 27 and 31, respectively) were selected for the treatment. Following amputation through the middle of the tail, tadpoles were exposed to a dose of 25mM hydrogen peroxide per day for 1, 3 and 7 days, respectively in three separate sets (Table 1). Each set comprising 100 tadpoles, was reared until the emergence of fore limbs. After the emergence of fore limbs tadpoles were fixed with 10% formaldehyde and processed for standard bone and cartilage staining.

**Results and Discussion**

Out of the three varieties of hydrogen peroxide tested in the present experiment, cum-H₂O₂ is found to be highly toxic in comparison to tert-H₂O₂ and H₂O₂ since all tadpoles died with in 24hr of exposure. On the other hand, induction of abnormal limb formation in tadpoles can also be achieved by tert-H₂O₂ indicating a lack of stringency for H₂O₂. However, the incidence of abnormal limb formation was higher with H₂O₂ than with tert-H₂O₂ (Table 1) for same concentration implying H₂O₂ as a better agent in inducing abnormal limb development. The most interesting was the development of specific type of abnormal limbs (Fig. 1B) in both the treated groups and limb abnormality was independent of exposure period (Table 1). There was no inhibition of tail regeneration. However, development of tadpoles was retarded as metamorphosis was delayed in the treated group (Table 1). Treated tadpoles were always stouter than the normal ones (Fig. 1A,B). The effect was stage specific since the hind limb bud stage tadpoles only developed the abnormal limbs (Fig. 2A). The pre
limb and limb paddle stage tadpoles showed no abnormal limb development.

Alcian blue and alizarin red stained limbs showed that the skeletal elements were all cartilaginous in nature (Fig. 2A-D). In abnormal hind limb, the femur, tibio-fibula and astragalus-calcaneum were all truncated with a bend at the middle and were stouter than those in the normal hind limbs (Fig. 2A, B). The proximal pieces of all the five digits were also bent in the middle (Fig. 2A, arrow). The inward curvature of all the digits of the abnormal hind limb was more in comparison to the normal hind limb. In the fore limb also all the skeletal elements were thicker than the normal fore limb (Fig. 2C, D). The humerus was normal but the radio-ulna was bent inwardly. Like the hind limbs the proximal piece of all the four digits of fore limbs were also bent at the middle. In contrast, no remarkable abnormality was observed in other skeletal elements including the skull, vertebral column, pectoral girdle and pelvic girdle.

Although it is clear that present added concentration of H₂O₂ in vitro can invoke a specific abnormal development and differentiation in limbs of tadpoles, the molecular mechanism that underlies these effects are not clear and needs further investigation. The possibilities of mediation of above effect of H₂O₂ through activation of transcription factors can not be ignored since H₂O₂ readily passes through the biological membrane and is shown to activate certain transcription factors, which have some relevance to growth responses. The abnormal limbs developed may be due to influence of H₂O₂ on activation of transcription factors including NF-kB. In chicks aberrant limb morphogenesis has been reported by inhibiting the nuclear transcription factor Kappa B (NF-kB). In the present experiment, pre limb bud, limb bud and limb paddle stage of tadpoles were treated. Development of limb was normal in pre limb bud and limb paddle stage tadpoles. Only the limb bud stage tadpoles were sensitive indicating a stage specific effect of H₂O₂. However, the patterning lay out of both the fore and hind limbs were not affected because each limb consisted of proximo-distal sequential portions. But, there was abnormal differentiation in the length (proximo-distal) of the limb as well as orientation of skeletal elements (bending). In addition, shortening of

| Table 1—Effect of daily dose (25 mM/ml) of hydrogen peroxide (a) and tertiary butyl hydrogen peroxide (b) on the limb bud stage tadpoles of P. maculatus |
|-----------------|---|---|---|---|
|                  | Control | 1day | 3days | 7days |
| Normal limb      |          |     |      |      |
| a                | 100%     | 20  | 10   | 20   |
| b                | 100      | 70  | 70   | 80   |
| Truncated limb   |          |     |      |      |
| a                | 0        | 80  | 90   | 80   |
| b                | 0        | 30  | 30   | 20   |
| Time (mean)      |          |     |      |      |
| for emergence    |          |     |      |      |
| of forelimbs     | 36       | 58.83 | 60.24 | 56.93 |
| (days)           |          | 60.31 | 59.36 | 60.85 |
| **Percentage of tadpoles** |

![Fig.1](A): Normal tadpole of *Poeciliopsis maculatus* (X2). (B): Hydrogen peroxide (left and middle) and tertiary butyl hydrogen peroxide (right) treated tadpoles of *Poeciliopsis maculatus* showing specific limb abnormalities (X2).
muscular elements in the thigh, shank and ankle of the hind limb was observed (Fig 2A). The skeletal differentiation was not affected but the center of the skeleton seemed to have been specifically affected leading to dorsal bending. Although, the tadpoles were exposed for a short duration at hind limb bud stage, the effects are evident much later during differentiation of the limbs. This indicates specific long-term effect of the treatment. It will be of interest to unveil the path through which hydrogen peroxide, a reactive oxygen intermediate (ROI) leads to specific abnormal limb development.

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References


