Effect of gibberellin-A$_3$ on metamorphosis in the Egyptian toad *Bufo regularis*

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KEYWORDS
Gibberellin A$_3$; *Bufo regularis*; Metamorphosis; Apoptosis

Abstract The present study was carried out to study the effect of gibberellin-A$_3$ on the metamorphosis of the Egyptian toad *Bufo regularis*. Samples of *B. regularis* were collected from Shebin El-Kom, Menoufia Governorate during the breeding season and kept in aquaria with little water till spawning, fertilized eggs are divided into 2 groups of 200 eggs each. The 1st group was treated with 5 ppm of gibberellin-A$_3$ (Berelex) 3 days/week for the period of metamorphosis. The second group was left untreated under the same environmental conditions and considered as a control. Hatchability of the eggs in each group was recorded. External lengths of the total body, hind limbs and the tail were measured. As a measure of apoptotic DNA fragmentation, the presence of DNA ladder was determined. The results showed that out of 200 eggs 47.5% were hatched in the control group and 76.5% were hatched in gibberellin-A$_3$ treated group. The average duration of metamorphosis was 60 days and 52 days in the control and treated groups, respectively. There was an increase in the total length of the hind limbs of the treated tadpole as compared with the control throughout the experimental periods. DNA isolated from control and tadpoles treated with gibberellin-A$_3$ showed degradation into oligonucleotide fragments forming a clear laddering pattern of apoptosis, but the treated tadpoles showed an increase of DNA fragmentation.

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Introduction

Plant growth regulators are chemical substances which are found to stimulate or to inhibit plant growth. Auxins, indolyle 3-acetic acid and gibberellins are representatives of these plant regulators. They are found to promote plant cell division and favor DNA synthesis (Audus, 1972; Abu-Sina and El-Shershaby, 1976). It is worth mentioning that the plant is the main source of animal food and it is very interesting from the biological point of view to study the effects of these substances on the animal tissues.

Abu-Sina and El-Shershaby (1976) showed that indolyl 3-acetic acid (IAA) increased the number of fibroblast cells cultivated in the calf serum. Also, the protein content of these cells was increased in the treated dishes on comparing with the control. In addition, an increase in the nitrogen content of the blood plasma in rats treated with IAA was reported by Abu-Sina and El-Shershaby (1980). This supports the view that this substance is capable of replacing tryptophane in the...
The precursors of the nucleic acids were reported, by some authors, to be affected by plant hormones. Farrow et al. (1976) showed that the plant auxins promote the incorporation of uridine into the nucleic acid of human leucocytes. Indolye-3-acetic acid is found in human urine and gibberellic acid-like substances are identified in the tissues of a wide variety of animals such as earth worms, chick embryos, cod fish sperm and human saliva (Audus, 1972).

The effect of gibberellic acid on animals was studied by some investigators. Carlisle et al. (1963) induced molting in larvae of both Locusta migratoria and Schistocerca gregaria by gibberellic acid. They added that the effect of gibberellic acid is similar to that of the insect growth hormone, ecdysone. El-Mofty (1973) induced cyst formation in the parasitic protozoan Opalina sudaficana by injecting its host Bufo regularis with ecdysone. The same author mentioned that the presence of cysts in therectum of toads injected with ecdysone is an indication that sexual reproduction took place in these parasites. El-Mofty (1974) obtained the same result in these parasites by injecting their hosts B. regularis with gibberellic acid. Anderson et al. (1982) reported that gibberellic acid had an androgenic effect in the chicken. Sakr et al. (2009) reported treating slugs Agriolimax reticulates with gibberellin-A3 stimulated gametogenesis. They added that gibberellin-A3 has an androgenic effect in this slug. Furthermore, feeding toads B. regularis with gibberellin-A3 was found to induce hepatocellular carcinomas in 16% of the studied animals (El-Mofty and Sakr, 1988). The results of the above mentioned authors indicated that gibberellic acid affected the growth of plants and animals through its action on cell division.

The metamorphosis of amphibians is the subject of numerous investigations which dealt with its control, especially the hormonal control. The present study was carried out to study the effect of gibberellin-A3 on metamorphosis of the Egyptian toad B. regularis.

**Materials and methods**

Samples of B. regularis were collected from Shebin El-Kom, Menoufia Governorate during the breeding season and kept in aquaria with little water till spawning. The gelatinous ribbons containing the fertilized eggs were spread in aquaria containing tap water and provided with Elodea for aeration. When tadpoles begin to feed, cooked spinach was supplied in the rearing water which was sufficient to enable the animals to swim freely.

At the beginning of the experiment, fertilized eggs are divided into 2 groups of 200 eggs each. The 1st group was treated with 5 ppm of gibberellin-A3 (Berlex) 3 days/week for the period of metamorphosis. The second group was left untreated under the same environmental conditions and considered as a control. Hatchability of the eggs in each group was recorded. External lengths of the total body, of hind limbs and of the tail were measured by a calibrated ocular micrometer and a binocular stereomicroscope.

For histological examination of thyroid glands, older tadpoles were decalcified in alcoholic HNO3 to soften their body skeleton. After fixation in Bouin’s fluid, specimens were dehydrated, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin. The diameter of the follicles and length of the follicular cells were measured by a calibrated ocular micrometer.

**DNA fragmentation assay**

As a measure of apoptotic DNA fragmentation, the presence of DNA ladder was determined according to Wlodek et al. (1991). Ten milligrams of testicular tissue was lysed in eppendorf tubes with 600 μl buffer (50 mM NaCl, 1 mM Na2EDTA, 0.5% SDS, pH 8.3) and gently shaken. The mixture was incubated overnight at 37°C then, 20 μl of saturated NaCl was added. The sample was shaken and centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to new eppendorf tubes and then DNA was precipitated by 600 μl cold isopropanol. The mixture was inverted several times till fine fibers appear, and then centrifuged for 5 min at 12,000 rpm. The supernatant is removed and the pellets were washed with 500 μl 70% ethyl alcohol, centrifuged at 12,000 rpm for 5 min. After centrifugation the alcohol was decanted or tipped out and the tubes plotted on Whatman paper to dry. The pellets were resuspended in 50 μl or appropriate volume of TE buffer (10 mM Tris, 1 mM EDTA, PH 8). The resuspended DNA was incubated for 30–60 min with loading mix (Rnase + loading buffer) and then loaded into the gel wells.

**Agarose gel electrophoresis**

A gel was prepared with 2% agarose containing 0.1% ethidium bromide (200 μg/ml). The DNA samples were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole FF and 30% glycerol) and loaded into the wells (2 μg of DNA/lan) with a standard molecular-sized ladder marker (Pharmacia Biotech., USA). The gel was electrophoresed at a current of 50 mA for 2.5 h using the submarine gel electrophoresis machine. The DNA was visualized and photographed with illumination under UV light using a photodocumentation hood (Fisher Scientific, Pittsburgh, PA, USA) equipped with a Polaroid 667 film with an orange filter (Kodak, Rochester, NY, USA).

**Results**

Out of 200 eggs 85 eggs (47.5%) were hatched in the control group and 14 eggs (76.5%) were hatched in gibberellin-A3 treated group (Table 1). The average duration of metamorphosis was 60 days and 52 days in the control and treated groups, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of metamorphosis</th>
<th>No. of hatched embryos</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60 Days</td>
<td>95</td>
<td>47.5</td>
</tr>
<tr>
<td>GA3 group</td>
<td>52 Days</td>
<td>153</td>
<td>76.5</td>
</tr>
</tbody>
</table>
apparent effects became evident by the 35th day of treatment in which the mean increase in the total body length was estimated to be 20%. Data were incomparable after 56 days of treatment due to the absence of tail in the treated groups after 52 days as compared with control (60 days). Data in Table 2 indicated also that there is an increase in the total length of the hind limbs of the tadpole treated with gibberellins-A3 as compared with the control throughout the experimental periods. Thus, by 21st day of treatment, the increase was 28% and at the end of observations it reached 36.5%. Gibberellin-A3 in the used dose did not affect the length of fore limbs in both groups.

Histological examination of the thyroid gland at stage 56 showed that the thyroid gland appeared was formed of follicles, each consisting of cuboidal cells with colloidal substances occupying the central cellular cavities. The mean diameter of the follicles is 0.68 ± 3.5 μm and the mean length of the cells is 4.8 ± 1.3 μm. GA3-treated tadpoles showed an increase in the follicular diameter (73.5 ± 4.9 μm) and the cells appeared columnar with a mean length 5.8 ± 1.8 μm (Table 3).

Biochemical features of apoptosis

DNA isolated from control and treated tadpoles with gibberellin-A3 showed degradation into oligonucleotide fragments forming a clear laddering pattern of apoptosis (Fig. 1). In the treated tadpoles, an increase of DNA fragmentation was recorded. The optical density at 800 bp was 21.8 in gibberellin-A3 treated tadpoles compared with 8.4 in controls.

Discussion

The present study revealed that gibberellin-A3 affects the metamorphosis of *Bufo regularis*. The treated tadpoles complete their metamorphosis faster than controls. Besides, gibberellin-A3 induced an increase in the length of hind limbs. Many investigations reported that limb development in amphibian is linked with and parallel to the thyroid development (Etkin, 1968; Dodd and Dodd, 1976). During this work, it is also found that the rate of tail resorption in treated animals is increased as compared with the controls. The process of tail resorption is induced by thyroid hormone (Weber, 1967; Etkin, 1968; Frieden and Just, 1970). At the biochemical level, tail atrophy has been found to coincide with a marked increase in the activities of acid phosphatase and other acid hydrolases (Weber, 1967) which are apparently involved in tissue destruction. The results showed that the diameter of thyroid follicles increased in GA3-treated tadpole together with an increase in the length of follicular cells and change their shape to columnar. These results were considered as evidence of increased activity of thyroid gland (Michael and Nour El-Din, 1991).

Apoptosis appeared in control tadpoles and increased after treatment with gibberellin-A3. Apoptosis plays an important role in many developmental processes such as cell differentiation, e.g. oogenesis (Sommer et al., 1998), organogenesis, and establishment of body structures (Jacobson et al., 1997; Sanders and Wride, 1995). Extensive morphological, cytological and biochemical analyses have shown that apoptosis is an essential aspect of many changes that occurred during amphibian metamorphosis. Kerr et al. (1974) examined electron microscopically the resorption of the tail muscle and epidermal cells during metamorphosis of the dwarf tree frog *Litoria glauerti*. They found that the two major cell types, epidermal and muscle cells, of the tail undergo a series of well defined, sequential morphological changes of apoptosis, including the condensation of the cytoplasm and nuclear chromatin and subsequent formation of the apoptotic bodies. Similar findings have also been reported for *Rana japonica* (Kinoshita et al., 1985) and *Xenopus laevis* (Nishikawa and Hayashi, 1995). Tata

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of gibberellin-A3 on total body length and length of hind limbs of tadpoles.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean length of hind limb (mm)</td>
<td>Mean of total body length (mm)</td>
</tr>
<tr>
<td>GA3-group</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0.5</td>
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<td>4</td>
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</tr>
<tr>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>6.3</td>
<td>4</td>
</tr>
<tr>
<td>6.33</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Days of complete absence of tail.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Effect of gibberellin-A3 on diameter of thyroid follicles and length of follicular cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Mean diameter of thyroid follicles (μm)</td>
</tr>
<tr>
<td>Control</td>
<td>68.2 ± 3.5</td>
</tr>
<tr>
<td>GA3 group</td>
<td>73.5 ± 4.9</td>
</tr>
</tbody>
</table>

* Significant at $P < 0.05, n = 15$. 

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(1993) reported that resorption of the larval tail during anuran metamorphosis takes place by apoptosis.

The relationship between thyroid hormone and apoptosis during amphibian metamorphosis was investigated by several studies. Nishikawa and Yoshizato (1986) isolated epidermal cells from the tail of *Rana catesbeiana* tadpoles and they found that they die in vitro when cultured in the presence of thyroid hormone. Myoblastic cell line derived from the tadpole tail of *Xenopus laevis* was found to respond to thyroid hormone by undergoing apoptosis (Yaoita and Nakajima, 1997). Shi et al. (2001) reported that thyroid hormone regulate apoptotic tissue during anuran metamorphosis through thyroid hormone receptors which are DNA-binding transcription factors.

Gibberellin-A3 was found to induce cell division in plants and animals (El-Moffy, 1974; Taiz and Zeige, 1991). It is speculated that gibberellins-A3 act as thyroid hormone which in turn modified the process of metamorphosis and apoptosis in *B. regularis*.

**References**


