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CITRAL INDUCES SKELETAL ANOMALIES DURING CHICK EMBRYO DEVELOPMENT

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***Background*: impairment of retinoic acid (RA) results in different malformations during embryonic development. Citral can interfere with the embryogenesis due to its direct inhibitory effect on RA. *Purpose:* is to evaluate the effect of different concentrations of citral on the skeletal elements of the developing chick embryo. *Methods:* Fertilized eggs of the chick *Gallus domesticus* were divided into control and experimental groups which received either three different concentrations of citral (50 µM, 100 µM and 200 µM). *Results:* treatment with citral caused several cases of scoliosis, absence of ossification in the skeletal system, deformed parrot beak, short and wry necks. Alizarin transparencies detected curvatures of the vertebral column at the cervical region. *Conclusion:* It is suggested that citral treatment in the present work caused inhibition of RA which might disrupt the expression of some genes, such as *BMPs, Fgf*8, *Msx1* and *Msx2* resulting in induction of morphological malformations. Citral induced morphological malformation in a concentration dependent manner. Cosmetics, food stuffs, detergents, flavoring agents and scenting agents that contain retinoic acid or citral are not recommended for ladies during the first trimester of pregnancy.**

Key words: citral - scoliosis - parrot beak - wry neck - ossification - chick embryo.

INTRODUCTION

Vitamin A is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinyl esters and several provitamin A carotenoids (most notably beta-carotene). Vitamin A has multiple functions: It is important for growth and development, for the maintenance of the immune system and good vision. Two forms of vitamin A are available in the diet: Preformed vitamin A (retinol and retinyl ester) and provitamin A (carotenoids). Both forms of vitamin A are intracellularly metabolized to retinal (retinaldehyde) and RA; the active form of vitamin A [1]. Many different diseases occur due to several physiological, chemical and morphological changes because of vitamin A deficiency. The World Health Organization recognizes vitamin A

deficiency as one of the major food problems affecting developing countries [2].

RA is a differentiation factor [3], in which the alcohol group is oxidized. RA is created at the brain [4]. RA is an endogenous molecule in embryonic vertebrates and adults. It plays an important morphogenetic role in most of the developing embryo systems..Retinal formation as a result of retinol oxidation is supposed to be the first step in the RA-generating pathway. Retinol or provitamin A is ingested and absorbed through the intestine, transported by retinol-binding proteins and stored in the liver [5]. Liver is the most important tissue for storing and metabolising vitamin A, and is responsible for systematization the homeostasis of retinoids, which is accomplished by the enzymes and proteins embroiled in the transport and metabolism of retinoids. In adult healthy individuals, about 90% of body vitamin A is stored in the liver, and is at most concentrated in lipid droplets of retinyl ester in hepatic stellate cells (HSC) [6]. The ability of vitamin A to influence development has been made possible by the enzymes that control the diversion of alcohol from retinol to aldehyde (retinaldehyde) and then to carboxylic acid (RA).

Retinol can be relocated to embryonic plasma by means of maternal plasma or egg yolk, depending on the species. In embryonic plasma, the lipophilic retinol fastens to retinol binding protein 4 (RBP4). Stra6 (stimulated by RA6) is a membrane receptor for RBP4; thus, retinol enters a cell where it is bound to one of the cellular retinol binding proteins (CRBP-I, CRBP-II, or CRBP-III). Retinol can be biologically transformed in a diversity ways, one of which is to be oxidized to more active retinoid forms. In contrast to the extracellular pathway of retinol transport, it can be an anabolic output of intracellular β-carotene metabolism resulting from either beta, β-carotene 15, 15´-monooxygenase 1(Bcmo1) activity, which produces two molecules of retinaldehyde that can be either reduced to retinol or oxidized to RA, or by asymmetric cleavage via β-carotene 9´,10´-dioxygenase 2 (Bcdo2) to form products that can be biotransformed to RA without formation of retinaldehyde [7]. The first step of RA synthesis, retinol oxidation to retinaldehyde, is spurred by many alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs). Genetic studies mention that at least three ADHs (ADH1, ADH3 and ADH4) and two RDHs (RDH1 and RDH10) play a physiological role in RA synthesis. Expressing these oxidizing enzymes for retinol is rife and overlapping. The second step of RA

synthesis, retinaldehyde oxidation to RA, is spurred by three retinaldehyde dehydrogenases (RALDH1, RALDH2, and RALDH3), which manifest nonoverlapping tissue-specific patterns of expression during embryogenesis [8]. If RA is produced in the cytoplasm, it can be linked to one of the cellular RA-binding proteins (CRABP-I, CRAPP-II), which are members of the family of fatty acid binding protein [7]. RA oxidation, which leads to its decay, is performed by three cytochrome P450 enzymes (CYP), known as CYP26A1, CYP26B1, and CYP26C1 [8]. Once RA is synthesized in the cell, it enters the nucleus and establishes or modifies the pattern of gene activity by binding to activated nuclear transcription factors. There are two types of these transcription factors (retinoid receptors) [9], **(1)** the RA receptors (heterodimers) (RARα, RARβ, and RARγ) that fasten the RA form, known as all-trans RA (atRA) to RA response elements (RAREs), and **(2)** retinoid X receptors (homodimers) (RXRα, RXRβ and RXRγ) that fasten the 9-cis-RA isomer to retinoid X response elements (RXREs) [7], as shown in the following diagram:

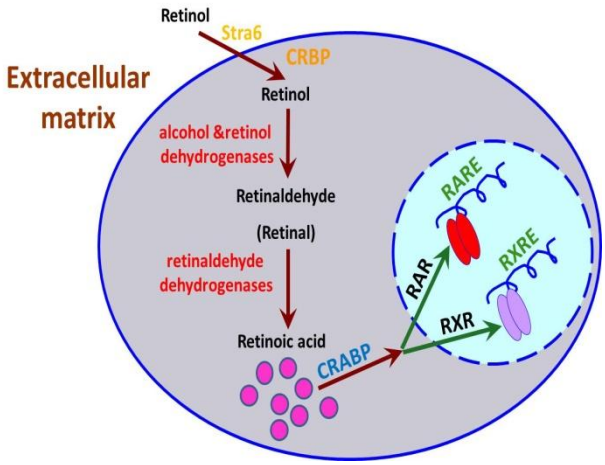


Figure (A): Illustration of biosynthesis and metabolism of RA

In chicks, RARα, RXRβ, and RXRγ were discovered in the mesenchyme of the neural crest that emigrates from the rostral neural tube to the facial primordia. These receptors are groups from the steroid thyroid hormone nuclear receptors superfamily [10], which fasten with specific DNA binding sequences found in the promoters of the RA-responsive genes, called the RA response elements (RARE). Such a link of RA-nuclear receptors complexes to RARE promoter elements leads to

initiate the transcription of genes whose protein mediates RA-biological responses [11]. Retinoid receptors are differentially expressed in embryonic and adult tissues.

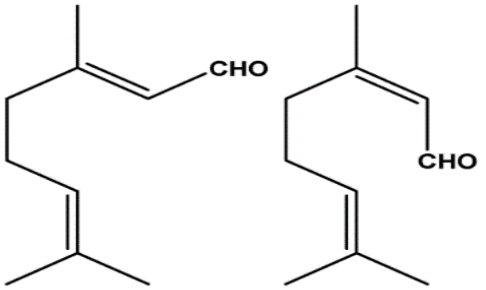
RA is a low molecular weight (300 Da), signaling molecule that is known to affect pattern formation in vertebrate embryos including limb outgrowth and axial patterning [12]. It is an essential ingredient of cell-cell signaling during vertebrate organogenesis.

Genes inclusive of fibroblast growth factors (*Fgfs*), Sonic hedgehog (*Shh*), the *Hoxa* complex the *Hoxb-8*, the *Hoxd* complex, the bone morphogenetic protein (*BMPs*), the muscle segment genes (*Msx*), *Wnt*-7a and *Lmx*-1 are known to be involved in the process of embryonic development and morphogenesis. It is generally assumed that RA is involved in instigating of development and morphogenesis implicitly in the induction of some of these genes, but not maintaining the expression during later development [13]. RA is the upstream activator of the nearby identified genes *Meis1* and *Meis2*. RA promotes proximalization of limb cells and preserves the proximal *Meis* domain in the limb. The range of the RA synthesis and signaling, which initially span the entire lateral plate mesoderm, is transformed into proximal limb domains through the apical ectodermal ridge (AER) activity following limb inception. The fibroblast growth factor (FGF) is a key molecule answerable for this AER activity and is integrated into the proliferation of limb cells, with a specific function in promoting distalization if RA production and signaling was dampen [14].

Essential oils extracted from nominated kinds of plants contain sturdy antimicrobial, antifungal and antiparasitic activities. One of the most substantial active ingredients of these essential oils responsible for such activities was proved to be citral [15]. Citral is found in the volatile oils of sundry herbal plants. It is a principal component of essential oils extracted of lemongrass (*Cymbopogon citratus*), Melissa (*Melissa officinalis*) and Verbena (*Verbena officinalis*) [16,17,18]. It is mobile light yellow with a lemon-like aroma, less dense than water and insoluble in water. Because of their special odor, antimicrobial, antifungal and insecticide effects, as well as their low toxicity and low carcinogenicity, citral is classification is “Generally Recognized as Safe” substance [19].

Citral, (3,7-dimethyl-2,6-octadienal), is one of the most substantial members of the open chain monoterpenoids. It is volatile α,β-unsaturated

aldehyde. Chemically, natural citral is a mixture of isomers, trans-isomer "geranial" (40 to 62%) and cis-isomer "neral" (25 to 38%) that have the same molecular formula, $C_{10}H_{16}O$ but having different structures [20]. Their chemical structures are:



citral – a (Geranial) (***trans*** **form)** **citral – b (Neral)** (***cis*** **form)** [20]

Due to its characteristic strong lemon-like odor and bitter sweet taste, citral is commonly used as food additive, as fragrance in the cosmetic industry, as a taste enhancer, as an odorant in perfumes and as an insect repellent [18].

Researchers clearly recognized the importance of vitamin A and its derivatives to the normal life of different animals. Its deficiency and surplus may cause several physiological, chemical and morphological changes in the animal body and as a result of these changes, many different diseases occur. Signs and symptoms of vitamin A deficiency are not easily ignored. Certain pathological conditions such as severe anemia, neurological lesions, night blindness and growth retardation results due to vitamin A deficiency. Clear evidences demonstrated that vitamin A deficiency (VAD) contributes to mortality from diarrheal diseases and measles, accounting for 1.7% of all mortality in babies below-five in developing nations [21]. There are several fetal abnormalities produced in the embryonic vertebrates, and resulting from VAD, including hindbrain segmentation defects, neural crest cell death, the absence of posterior branchial arches and anomalies of facial structures, limb buds, eyes and somites [22].

There are lots of evidences showed that citral can interfere with the embryogenesis and carcinogenesis [23]. Sundry studies have shown that citral is an important material in the manufacture of certain chemicals, such as vitamin A [24]. It can organize the synthesis of RA [25]. Citral is a generic alcohol dehydrogenase antagonist that is thought to interpose with retinoid synthesis [26]. It is a suppressor for retinaldehyde dehydrogenases [27].

Citral has been reported to display activity as a vitamin A antagonist by dampen the oxidation of retinal to RA [23]. It dampens both steps in RA synthesis from retinol, since it can act as a substrate for both the alcohol and aldehyde dehydrogenases. This suggests that citral is able to prohibit the endogenous RA signaling pathway [28], as shown in the following diagram:

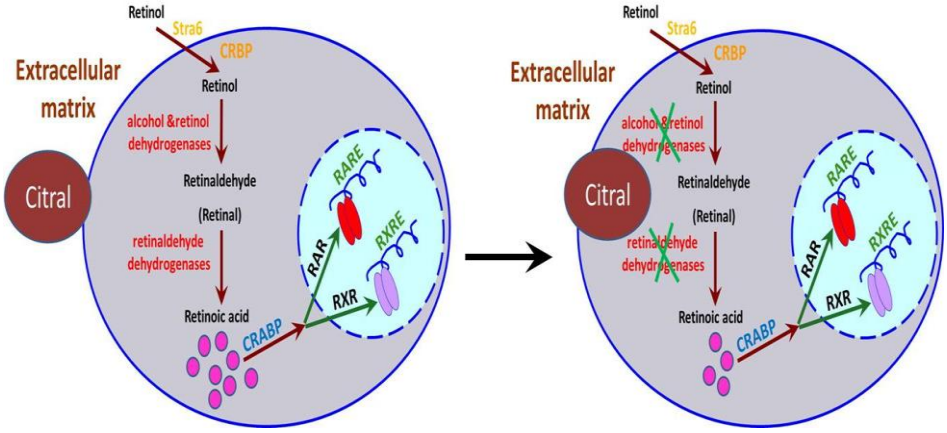


Figure (B): Citral inhibits synthesis of RA.

Di Renzo *et al*. [23] found that low concentrations of citral effectively prohibit the formation of RA in rats. In *Xenopus laevis* embryos citral inhibit the formation of RA and thus citral treatment can save embryos from the teratogenic effects of exogenous retinol [28]. The large number of craniofacial and central nervous system anomalies in citral-treated embryos complicates the relationship between diminished RA signaling and olfactory pathway development. The retinoid-mediated gene expression is prohibited in mouse embryos at midgestation with citral; the spectrum of morphogenetic anomalies includes disruption of olfactory

pathway development [26]. Song *et al.* [29] investigated late stages of chicken embryos and specifically targeted the nasal pit with citral. The researchers examined the effect of prohibiting the synthesis of retinoids on the development of craniofacial and conducted rescue experiments with RA to test the specificity of citral on retinaldehyde dehydrogenase. The result was a nominated loss of derivatives from lateral nasal prominences. Providing exogenous RA rescued development of the beak demonstrating that most of the defects caused by the citral were produced through specific prohibition of the RA synthesis.

MATERIAL AND METHODS

1. *Chick embryo:*

Fertilized eggs of the chick *Gallus domesticus* (*Dandrawi* strain), obtained from the farm of Faculty of Agriculture, Assiut University, were used in the experiments of the present investigation. All embryological materials needed for the experiments were obtained by artificial incubation using an electrical thermostatically controlled incubator. The incubator was located in a well-ventilated place and was accurately adjusted at 37.5± 0.1°C before use. Both the trays of the eggs and inside of the incubator were thoroughly cleaned using dettol and ethyl alcohol. Sterilization was carried out using Biocidal ZF reagents from Wak-Chimie Germany. Labeled fertile eggs were placed vertically in the trays inside the incubator. Ventilation was allowed in the incubating chamber. Relative humidity was automatically adjusted at 52%. Incubated eggs were automatically turned approximately bihourly from side to another until their operation time. The incubator used in the present study belongs to PTO, Egypt, model C5.

2**. *Experimental design:***

The incubated eggs were randomly divided into 4 groups (35 eggs each):

1. The first group: was left untreated as a control one.
2. The second, third and fourth groups: received one injection per embryo each one was 100 µl of different doses of citral (Sigma), i.e. 50 µM, 100 µM, and 200 µM respectively, in saline solution.

All the injections were carried out just before incubation. Eggs were thoroughly cleaned with alcohol. A hole was done at the blunt area of the egg. Injection was carried out by means of a Hamilton microsyringe. The needle was inserted vertically for a suitable distance into the yolk sac. The hole was then sealed with a sealing tape. The eggs were incubated until

they were taken out at 18 days of incubation for obtaining the required embryonic stages.

3. *Specimens' preparation:*

The eggs were carefully opened under physiological saline solution. Embryos were carefully removed from the yolk and membranes and they were transferred to a new saline solution for washing and then fixed in 10% neutral formalin and 95% ethyl alcohol. Specimens were morphologically examined. To investigate the skeletal elements, transparencies of the body were prepared by using Alizarin Red S stain according to the modified method by Staples & Schnell [30].

4. *Statistical analysis:*

The percentages of the beak and neck deformities were calculated and statistically analyzed using column statistics and one-way analysis of variance with the Newman–Keuls multiple comparison test as a posttest. These analyses were carried out using Excel (Microsoft office 10).

RESULTS

Control group:

At the age of eighteen days, this age has nearly all characteristic features of complete development. The body increased in length. The eyes remain partially closed. Auditory opening and external naris are more developed than younger ages. The beak became more developed. Limbs reached the adult form except for size. Toes have fine horny scales ending with claws (Fig. 1). At this stage, alizarin transparency revealed ossification of all skeletal elements including beak and phalanges of toes (Fig. 2).

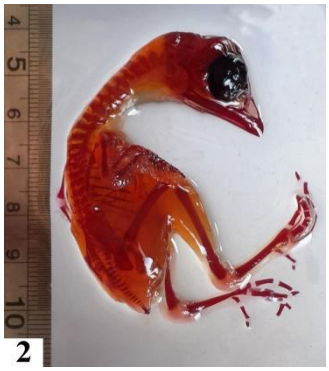
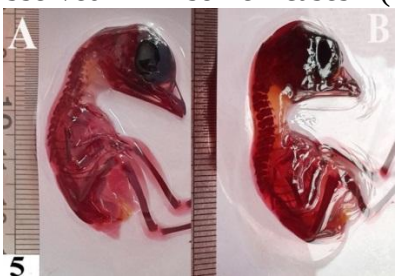


Figure 1: A photograph of a control chick embryo eighteen days after incubation.

Figure 2: A photograph of alizarin preparation of a control chick embryo eighteen days after incubation, showing ossification of skeletal elements of all body parts.

50 µM citral-injected group:

Some of the studied specimens showed incompletely closure for the upper and lower halves of the beak (Fig. 3). The neck was short in a case (Fig. 4), when such a case was demonstrated with alizarin transparency; it revealed a curvature of the vertebral column at the end of cervical region (Fig. 5). Other case with alizarin transparency revealed a long neck (Fig. 6). A wry neck was also observed in some cases (Fig. 7)



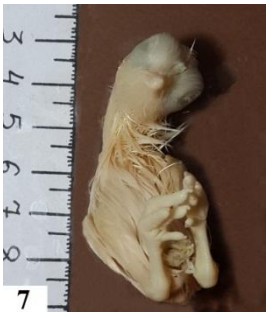


Figure 3: A photograph of a chick embryo head treated with 50 µM citral eighteen days after incubation, showing incompletely closed upper and lower halves of the beak.

Figure 4: A photograph showing a chick embryo treated with 50 µM citral eighteen days after incubation, with short neck.

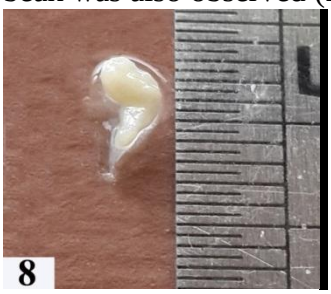
Figure 5: A photograph of alizarin preparation of a chick embryo treated with 50 µM citral eighteen days after incubation, showing curvature of the vertebral column at the end of cervical region (A&B).

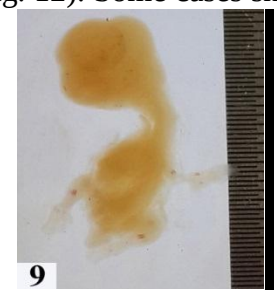
Figure 6: A photograph of alizarin preparation of a chick embryo treated with 50 µM citral eighteen days after incubation, showing a long neck.

Figure 7: A photograph of a chick embryo treated with 50 µM citral eighteen days after incubation, showing wry neck.

100 µM citral-injected group:

The studied cases showed scoliosis (Fig. 8). Upon demonstration of a case with alizarin transparency, it revealed absence of ossification in the skeletal system, except for little elements in fore and hind limbs (Fig. 9). The beak also exhibited several malformations such as, the upper jaw was longer than the lower one (Fig. 10). In some cases the upper half of the beak was relatively pulled away from the lower one (Fig. 11). A parrot beak was also observed (Fig. 12). Some cases showed wry neck (Fig. 13).





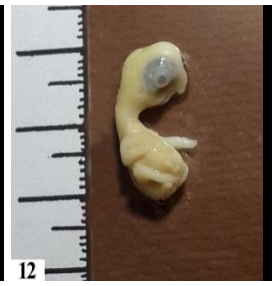


Figure 8: A photograph of a scoliotic and retarded chick embryo treated with 100 µM citral eighteen days after incubation.

Figure 9: A photograph of alizarin preparation of a chick embryo treated with 100 µM citral eighteen days after incubation, showing absence of ossification except for little limb elements.

Figure 10: A photograph of a growth retarded chick embryo treated with 100 µM citral eighteen days after incubation, showing elongation of the upper jaw than the lower one.

Figure 11: A photograph of a head of a chick embryo treated with 100 µM citral eighteen days after incubation, showing upper half of the beak pulled away from the lower one.

Figure 12: A photograph of a chick embryo treated with 100 µM citral eighteen days after incubation, showing parrot beak.

Figure 13: A photograph of a chick embryo treated with 100 µM citral eighteen days after incubation, showing a wry neck.

200 µM citral-injected group:

Many cases showed scoliosis (Fig. 14). In some cases, the upper half of the beak was longer than the lower one (Fig. 15), while in other cases, the upper half of the beak was shorter than the lower one (Fig. 16). The parrot beak was observed in some cases (Fig. 15). A wry neck was also observed in some cases (Fig. 17). When such a case was demonstrated with alizarin transparency, it revealed a curvature in the cervical vertebrae (Fig. 18). Sometimes the neck was shorter than control. Upon demonstration with alizarin transparency, it was found that the configuration and interference between the cervical vertebrae was different from that of the control (Fig. 19).

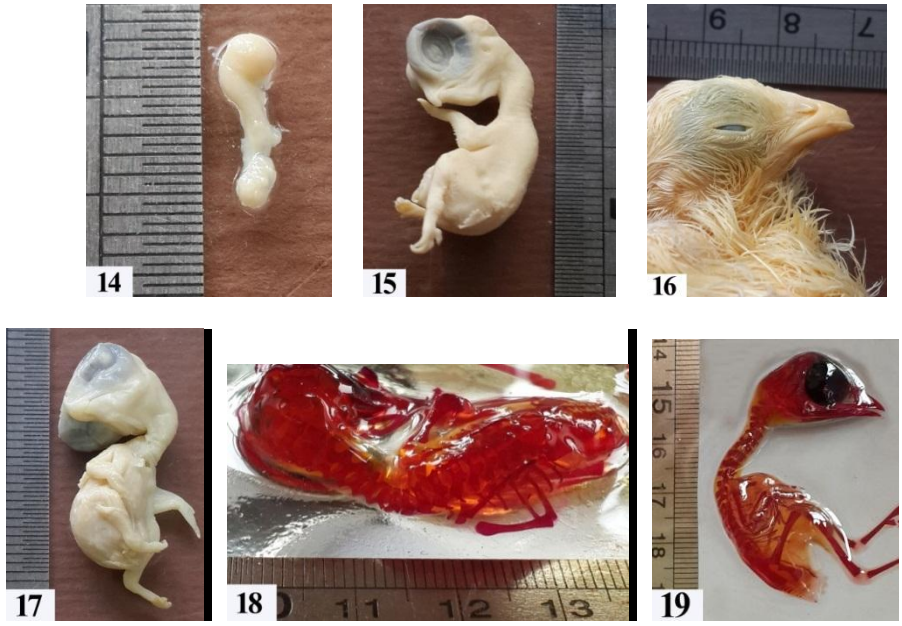


Figure 14: A photograph of a growth retarded chick embryo treated with 200 µM citral eighteen days after incubation, showing scoliosis.

Figure 15: A photograph of a growth retarded chick embryo treated with 200 µM citral eighteen days after incubation, showing parrot beak with long upper half.

Figure 16: A photograph of a head of a chick embryo treated with 200 µM citral eighteen days after incubation, showing the short upper half of the beak.

Figure 17: A photograph of a chick embryo treated with 200 µM citral eighteen days after incubation, showing wry neck.

Figure 18: A photograph of alizarin preparation of a chick embryo treated with 200 µM citral eighteen days after incubation, showing a curvature in the cervical vertebrae.

Figure 19: A photograph of alizarin preparation of a chick embryo treated with 200 µM citral eighteen days after incubation, showing short neck with misconfigured cervical vertebrae.

Statistical analysis:

Statistical analysis revealed that the deformities of beak and neck were elevated with the increasing citral concentrations (50 µM, 100 µM and 200 µM) (Fig. 20).

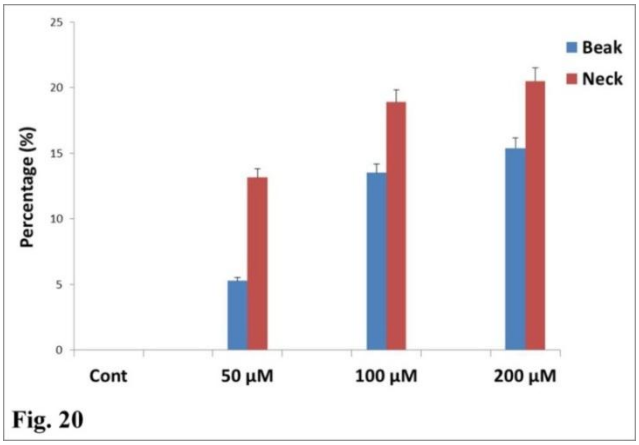


Figure. (20): The percentages of the beak and neck deformities. A comparison between control and three different concentrations of citral (50 µM, 100 µM and 200 µM).

DISCUSSION

In the current study, several cases of scoliosis, absence of ossification in the skeletal system, deformed parrot beak, short or wry necks were observed. Alizarin transparencies disclosed curvatures of the vertebral column at the cervical region.

Some clues come from studies on skeletal tissues, where retinoid signaling plays important roles in governing proliferation and differentiation of chondrocytes during endochondral bone formation and particularly in the regulation of *Bmp*s signaling during chondrogenesis and osteogenesis. *Bmp*s are well known to control chondrocytes and

osteocytes during the formation of endochondral bones, and *BMP*s are manifested in the development of the cranial base. In particular, *BMP4* boosts proliferation and hypertrophy in the developing cranial base. Retinoid signaling modulates proliferation, differentiation, programmed cell death, and expression of *BMP4* during cranial base development. Adding RA-soaked beads in cultures of cranial base samples reduced cartilage matrix and *BMP4* expression while hypertrophic chondrocytes has become very small and highly proliferative. Suppression of retinoid signals by applying citral, slightly fostered *BMP4* and induced programmed cell death in the chondrocytes within the sphenooccipital synchondrosis, while hypertrophy of chondrocytes was retarded [31].

Lack of the adequate quantities from RA and *BMP4* needed during gestation resulted in a lot of birth defects including craniofacial deformities. Koussoulakou *et al*. [32] explored the effect of RA and *BMP4* signaling antagonists (citral and anti-BMP4 antibodies) on fetal mouse head and tooth morphogenesis, discrimination and apoptosis. Their data indicated that administration of citral caused restriction/retardation of cranial chondrogenesis and osteogenesis. Apoptosis was not detected in teeth tissues. Management of anti-BMP4 antibodies resulted in temporary interference with the normal pathway of odontoblast discrimination and the production of pre-dentin. Animals at the adulthood displayed a fairly normal phenotype. Levels of retinoids are one signal required for establishing frontonasal mass identity. In the embryonic face the nasal pit is a substantial source of retinoids. Creating retinoids are required in the nasal pit to develop lateral nasal prominence and sequent morphogenesis. When citral-steeped beads were immersed in the nasal pit of stage 20 chicken embryos, the result was a specific absence of derivatives from the lateral nasal prominences. Providing exogenous retinoic acid rescued development of the beak indicating that most of the defects caused by the citral were produced by specific prohibition of RA synthesis. Increased levels of retinoid acid joint with prohibition of *BMP* signals led to the transformation of the side of the beak (derived from the maxillary prominence) till a second collection of midline structures (derived from the frontonasal mass). These results revealed that it is necessary to have a source of endogenous retinoids to pattern the frontonasal mass and its derivatives, the prenasal and premaxillary skeletal elements. The effects of citral beads on skeletal morphogenesis were harmonious with early changes in nasal pit morphology. Prohibited RA synthesis decreased the expression of *Msx1, Msx2,* and *Bmp4*. Citral stopped the upregulation of

*Fgf*8 over the lateral edge while it was expressed on the medial side, and this may have participated to the specific increase in apoptosis in lateral nasal prominence. Those data evinced that endogenous retinoids work in the upstream direction of *Fgf*8 and that the equipoise of these two factors is crucial in regulating programmed cell death and facial morphogenesis [29].

The axial skeleton is originated from paraxial mesoderm, which undergoes a molecular timing process of somitogenesis to produce symmetrically arranged blocks of tissue (somites) on each of the midline neural tube sides and notochord. The process of somitogenesis proceeds as follows: the paraxial mesoderm flanking the axial neural tube and notochord is composed of loose mesenchyme. The expression of cyclically expressed genes of the *Notch/Delta* signaling system under the influence of RA, *Fgf*8 and *Wnt3a* acting within the paraxial mesoderm defines the posterior confines of the next somite to be formed. In a mesenchymal-epithelial transformation (MET), the loose mesenchyme of the forming somite condenses and forms an epithelial sphere. Such somitogenic MET is primarily accomplished by cadherin activation controlled by *Wnt* and *Fgf* signals emanating from the overlying ectoderm and inducing paraxis expression and epithelialization. The epithelial somite is maintained for a time by *BMP* signals from the neighboring neural tube. Later, the antagonism between notochordal *Shh* and *BMP-4* from the ventral neural tube induces the expression of *PAX1* in the ventromedial somitic quadrant or sclerotome, the chunk of the somite that gives rise to the axial skeleton. The expression of *PAX1* in the cells heralds an epithelial-mesenchymal transformation (EMT), in which they dissociate from each other and relocate themselves to the region surrounding the notochord and ventral neural tube. Here, the mesenchymal sclerotomal cells begin expressing *SOX9* and undergo chondrogenic differentiation to produce the ventral components of the cartilaginous vertebral anlage. The somite has a distinct anterior-posterior polarity. The posterior half of one somite amalgamates with the anterior half of the next posterior somite during the process of resegmentation. This resegmentation results in bony elements that are out of phase with the muscular and tendinous components, forming a vertebral joint and a flexible vertebral column. Scoliosis is one of the rife axial defects and is known as a curvature in the spine in the coronal plane due to structural deformation and defects of the vertebrae. These blemishes have their origin in the somitogenesis, the initial appearance of the vertebral

column's metameric segmentation. RA can also stimulate axial skeletal defects, including homeotic transformations and axial skeletal truncations. RA, its receptors and enzymatic activators are expressed in the paraxial mesoderm and act as crucial regulators in somitogenesis clock coordination and *Hox* gene expression [33].

Spatially-restricted expression domains of *Msx1* and *Msx2* in the developing chick face mention that they may play a role in epithelial-mesenchymal interactions that control the outgrowth of facial primordial. High levels of *Msx* gene transcripts in upper and lower beak primordia correlate with regions of outgrowth. RA treatment resulted in changes in *Msx1* and *Msx2* expression that caused rapid down-regulation in upper beak primordia where outgrowth was inhibited, but the pattern of *Msx* gene expression was essentially unchanged in lower beak primordia, which continued to grow out normally. Differential expression of molecules involved in the retinoid-response pathway could be important in mediating the sensitivity of certain primordia to the teratogenic effects of retinoids. In the developing chick face, the RARβ and CRAPP transcripts are expressed in the upper beak primordial, whilst in the lower beak primordia, the RARβ transcripts are expressed weakly but the CRAPP transcripts are expressed at higher levels than in the else facial primordia. Consequently, the preferential expression of these molecules mastery help mediate the localized retinoid created changes in the face and play a role in impress the expression of *Msx1* and *Msx2*, chiefly in upper beak primordia. This provides direct evidence to support the suggestion that RA causes the cleavage of the primary palate in chick embryos by inhibiting the expression of *Msx1* and *Msx2* in the growing facial primordial [34].

Shimomura *et al*. [35] checked whether RA signals regulated *Lhx8, Msx1* and *Msx2* transcription through *Fgf* signals in the maxillary prominence. Exogenous RA implementation caused severe defects of the maxilla. Prohibiting RA synthesis with Citral causes a specific loss of derivatives from the maxillary prominences. RT-PCR analysis of maxillary mesenchyme disclosed that the expressions of *Lhx8, Msx1 and Msx2* were significantly down-regulated by RA as well as by citral. The downregulated *Lhx8* was rescued through the co-treatment with *Fgf-8b*, which indicated a downstream of the RA signal. *Fgf-8b* induced up-regulated *Lhx8* expression while the SU5402, a pan-FGF family antagonist, down-regulated and causes maxillary morphogenesis

deformity and slit lip. The authors suggested that *Lhx8* is organized by RA signals by dint of *Fgf* signals and that RA and *Fgf-8b* levels can control the upper jaw morphogenesis.

Present data revealed that the increased citral concentration resulted in increased frequency of beak and neck anomalies. This means that citral induces morphological anomalies in a concentration manner. It is suggested that the deformations of the beak in the present work, including relative elongation or shortening of the upper or lower half compared to each other, or being pulled away from each other and appearance of several cases of parrot beak, might be due to low expression of *Msx1* and *Msx2* genes which are normally expressed in a gradient manner and are responsible for the upper and lower beak primordial. Treatment with citral in the current work caused repression of RA and that might obstruct the expression of such genes. With regard to recording of scoliosis, demise of ossification in the skeletal system, wry neck and the curvature of the vertebral column in some cases of the present work, it might be due to disruption of *BMPs* and *Fgf*8 signals. Prohibition of RA synthesis by citral resulted in decreased *Msx1, Msx2, Bmp4* expression and ban upregulation of *Fgf*8. Our results are in assent with the findings of the earlier studies aforesaid above. Cosmetics, food stuffs, detergents, flavoring agents and scenting agents that contain retinoic acid or citral are not recommended for ladies during the first trimester of pregnancy.

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السيترال يحفز التشوهات الهيكلية أثناء النمو الجنيني للكتكوت

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حامض الرتينويك، أحد المشتقات الطبيعية لفيتامين (أ)، هو عامل تمايز قوي. يلعب دورًا مهمًّا فى كثير من العمليات الحيوية خلال مراحل النمو الجنينى. السيترال هو مركب رئيسي من الزيوت العطرية المستخرجة من العديد من النباتات العشبية. يمكن أن يتداخل السيترال مع التطور الجنيني بسبب تأثيره المباشر والمثبط لحامض الرتينويك، ومن ثَمَّ كان الهدف من هذه الدراسة هو تقييم تأثير تركيزات مختلفة من السيترال على تَشَكُّل العناصر الهيكلية لجنين الكتكوت أثناء نموه.

استُخدِم فى هذا البحث بيضٌ مخصبٌ للكتكوت من نوع "*جالاس دوميستيكس*" سلالة الدندراوي. تم تقسيم البيض إلى مجموعات وهى: المجموعة الضابطة، ومجموعات تجريبية والتي تلقت ثلاثة تركيزات مختلفة من السيترال (٥٠ ميكرومولر ، ١٠٠ ميكرومولر و ٢٠٠ ميكرومولر).

أوضحت النتائج المورفولوجية أن حقن جنين الكتكوت بالسيترال تسبب في حدوث تأثيرات مشوهة قوية في الهيكل العظمي أثناء التَشَكُّل الجنيني. وشملت هذه التشوهات اختفاء التعظم فى بعض المناطق من الهيكل العظمي، تشوهات في المنقار و تقوسات في العمود الفقري وفقرات العنق. وقد ازدادت معدلات التشوهات بزيادة تركيز السيترال.

من المحتمل أن استخدام السيترال في هذه الدراسة قد تسبب في تثبيط حامض الرتينويك الداخلي وقد أدي ذلك إلى تعطيل واحداث خلل لبعض الجينات مثل *BMPs، Fgf8، Msx1 و Msx2* التي يتم التعبير عنها بشكل طبيعي فى بعض الأجزاء أثناء تكوينها. يُعتَقد أن تأثير السيترال يتبع نمطاً كمياً حيث أن زيادة تركيز السيترال أدت الى زيادة معدل التشوهات.

ولذلك لا ينصح للسيدات باستخدام مستحضرات التجميل والمواد الغذائية والمنظفات والمواد المنكهة والعطرية التي تحتوي على حمض الريتينويك أو السيترال خلال الأشهر الثلاثة الأولى من الحمل.