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Effects of maternal administration of aluminum chloride on the development of the skeletal system of albino rat fetuses - protective role of saffron

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SUMMARY

Aluminum is widely used in food packaging and additives. Aluminum chloride ($AlCl_3$) was known to cause maternal toxicity and embryolethality. Previous studies have demonstrated the antioxidant effects of saffron. The purpose of the present study was to assess the effect of maternal administration of aluminum chloride during the period of embryogenesis on the development of the skeletal system of albino rat fetuses and the protective role of saffron. Twenty four virgin female albino rats were used throughout this study. One male rat was introduced into a cage with two females for mating. Once the pregnancy was confirmed, pregnant rats were divided into the following groups: Control, $AlCl_3$ treated (200 mg/kg) and $AlCl_3$+S treated ($AlCl_3$ 200 mg/kg and saffron 200 mg/kg in water extract). Rats received treatments daily from the 6th to 15th day of gestation intragastrically and sacrificed on the 20th day. The fetuses were obtained through Caesarian section, stained with Alizarin red and examined for skeletal development. $AlCl_3$ treated rats showed toxic manifestations and decreased weight gain and their fetuses revealed increased embryolethality and a higher number of bones showed delayed ossification. $AlCl_3$ +S treated animals revealed improvement in maternal weight gain, embryolethality and bone ossification. We conclud that $AlCl_3$ induces delay in bone development, and saffron ameliorates its effects.

Key words: aluminum chloride – saffron – skeletal development

INTRODUCTION

Aluminum (Al) comprises about 8% of the earth's crust, and is found in combination with oxygen, silicon, fluorine, chloride and other elements in soil, rocks and clays (Sigel and Sigel, 1988). Aluminum salts are extensively added as coagulant agents in water purification processes (Domingo et al., 1993). Aluminum metal is used in the manufacturing of automotive aircraft, alloys, cooking utensils, decorations, fencing, highway signs, cans, food packaging, foil, dental crowns, antacid drugs, food additives, toothpaste, and some cosmetics (Yokel, 2000; Yumoto et al., 2001).

Aluminum, once absorbed from the gastrointestinal tract, it is distributed into most organs of the body. It accumulates mainly in bone at high-dose levels and passes the blood brain barrier. High concentrations of aluminum chloride were known to cause serious effects on several body functions (World Health Organization, 2003), as well as maternal toxicity, embryolethality and resorp-

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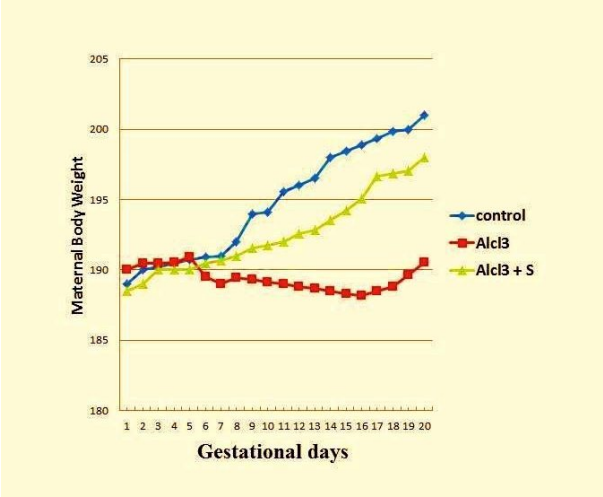


Fig. 1. Influence of aluminum chloride and saffron on maternal weight gain during gestation.

tion (Benett et al., 1975). Aluminum has been found to cause oxidative stress (Yousef, 2004) and potentiate the activity of iron ions Fe2+ and Fe3+ causing tissue damage (Newairy et al., 2009).

Saffron, the dried stigmata of the flowers of the saffron plant (Crocus sativus L., Iridaceae), is widely used as food flavoring and coloring agent. Also it is used in the manufacture of many medicines (Schmidt and Hensel, 2007; Kianbakht, 2009). Previous studies have demonstrated various pharmacological effects of saffron and its active constituents including anti-oxidant (Assimopoulou et al., 2005; Kanakis et al., 2007; Chen et al., 2008; Kianbakht and Mozaffari, 2009 and Ehsan et al., 2010), anti-tumor (Tavakkol et al., 2008), neuroprotective (Ochiai et al., 2007), analgesic and anti-inflammatory (Hosseinzadeh and Younesi, 2002), anti-convulsant (Hosseinzadeh and Khosravan, 2002), anti-anxiety (Pitsikas et al., 2008), bronchodilator (Boskabady and Aslani, 2006), antitussive (Hosseinzadeh and Ghenaat, 2006), and immune-stimulant (Kianbakht and Ghazavi, 2005).

The present study was carried out to assess the effect of maternal administration of aluminum chloride during the period of embryogenesis on the development of the skeletal system of albino rat fetuses, as well as the protective role of saffron.

MATERIALS AND METHODS

Dried red stigmas of saffron were obtained from a natural herb market in Ismailia City, Egypt. Water extract of saffron was prepared by immersion of 0.5 g of dried stigmas of saffron in 50 ml of distilled water for 24 hours, followed by filtration and kept in a clean and closed bottle (Ali and Saad, 2010). The water extract of saffron was prepared every 4 days. Aluminum chloride (AlCl3) was obtained from the Chemical Lab, Faculty of Medicine, Suez Canal University and it's solution was prepared by dissolving in distilled water.

Animals and breeding

Twenty four virgin female albino rats (weighing between 150 and 200 grams) were obtained from the animal house of the Faculty of Veterinary Medicine, Suez Canal University, and used throughout the present study. They were left at the experimental animal house for one week, allowing them to acclimatize to the new environment. All animals were housed in spacious wire mesh cages in a well-ventilated room, and received water and food *ad libitum*. All experiments were carried out

Table 1. Effects of prenatal administration of aluminum chloride and saffron on albino rat embryolethality.

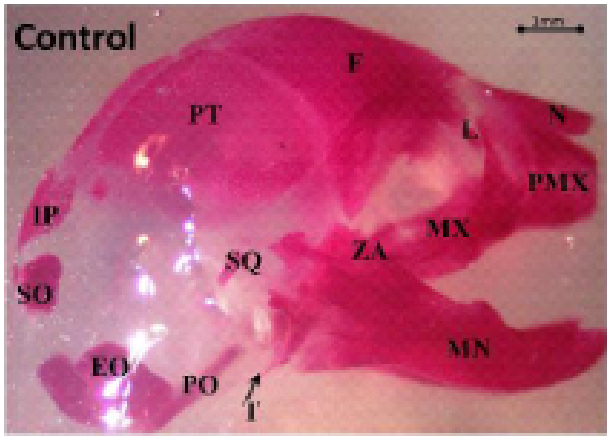
Groups	Total no. of implantations (metrial glands)	No. of resorption	No. of live fetuses	No. of dead fetuses
Control	60	0	60	0
$AlCl_3$	52	11 a, b	37	4 a
$AlCl_3$ + S	54	2	51	1

Chi-square test: a) P<0.05 compared to control, b) P<0.05 compared AlCl3 +S treated group

Table 2.- Effects of prenatal administration of aluminum chloride and saffron on craniofacial bone ossification in 20 day-old albino rat fetuses.

Bone ossification		Control (no. of bones)	$AlCl_3$ (no. of bones)	$AlCl_3$ + S (no. of bones)
Craniofacial bones	Complete	1588	828	1284
	Partial	28	520 a, b	160 a
	Absent	4	56 a, b	14 a

Chi-square test: [a] P<0.05 compared to control, [b] P<0.05 compared to AlCl3 + S treated group.



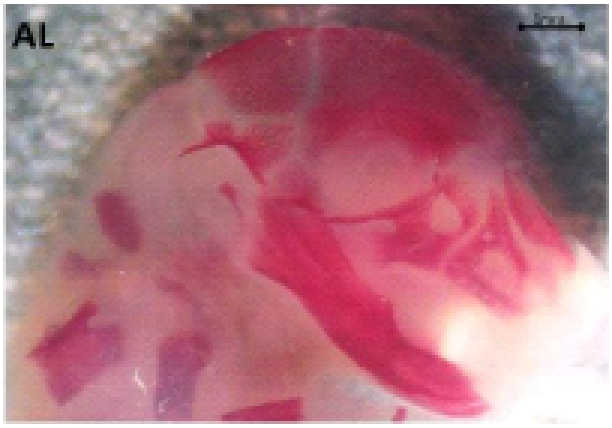


Fig. 2 (left). Lateral view of 20 day-old albino rat fetus from control group showing complete ossification of frontal (F), parietal (PT), interparietal (IP), supraoccipital (SO), exo- occipital (EO), basioccipital (PO), tympanic bulla (T), squamosal (SQ), zygomatic arch (zA), maxilla (MX), premaxilla (PMX), mandible (MN), lacrimal (L) and nasal (N) bones. x 35 (Alizarin red stain). Scale bar, 1 mm.

Fig. 3 (right). Lateral view of 20 day-old albino rat fetus from aluminum chloride treated group showing partial ossification of frontal, parietal, tympanic bulla, nasal, premaxilla, maxilla, squamosal and exo-occipital bones and absent ossification of interparietal, basioccipital and supraoccipital bones. x 30 (Alizarin red stain). Scale bar,1 mm.

in accordance with the guidelines of the Institutional Animals Ethics Committee. One male rat was introduced into a cage with two females and remained there over night. Pregnancy was detected by the presence of spermatozoa in the vaginal smears and this was considered as the first day of gestation (GD1).

Animal treatment

Once the pregnancy was confirmed, pregnant rats were divided into three groups (eight rats each) as follows: (1) Control group: rats were given distilled water, (2) Aluminum chloride treated group ($AlCl_3$): rats were received aluminum chloride at a dose of 200 mg/kg body weight (Mestaghanmi et al., 2002) and (3) Aluminum chloride + saffron treated group ($AlCl_3$+S): rats were received aluminum chloride (200 mg/kg)and water extract saffron (200 mg/kg) (Ali and Saad, 2010). The pregnant rats received treatments daily from the 6th to 15th day of gestation through

Table 3. Effects of prenatal administration of aluminum chloride and saffron on vertebral bone ossification in 20 day-old albino rat fetuses.

Bone ossification		Control (no. of bones)	$AlCl_3$ (no. of bones)	$AlCl_3$ + S (no. of bones)
Cervical arches (C1-C7)	**Complete**	420	347	370
	Partial	0	17 a	8 a
	Absent	0	0	0
Thoracic arches (T1-T13)	**Complete**	780	662	697
	Partial	0	14 a, b	5 a
	Absent	0	0	0
Lumbar arches (L1-L6)	**Complete**	360	276	313
	Partial	0	26 a, b	8 a
	Absent	0	19 a, b	3
Sacral arches (S1-S4)	**Complete**	60	10	42
	Partial	0	17 a, b	4 a
	Absent	180	181	170
Thoracic centra (T1-T13)	**Complete**	780	660	698
	Partial	0	16 a, b	4 a
	Absent	0	0	0
Lumbar centra (L1-L6)	**Complete**	360	283	314
	Partial	0	28 a, b	7 a
	Absent	0	10 a, b	3
Sacral centra (S1-S4)	**Complete**	235	181	212
	Partial	5	19 a, b	9 a
	Absent	0	8	1

Chi-square test: [a] $P<0.05$ compared to control, [b] $P<0.05$ compared to AlCl3 + S treated group.

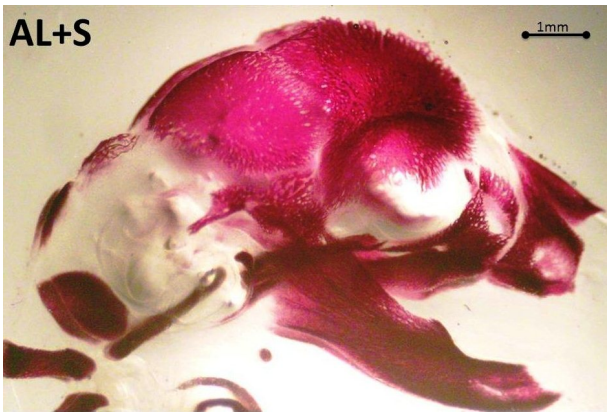


Fig. 4 (left). Lateral view of 20 day-old albino rat fetus from aluminum chloride and saffron treated group showing partial ossification of interparietal, supraoccipital, maxilla and premaxilla bones. x 30 (Alizarin red stain). Scale bar, 1 mm.

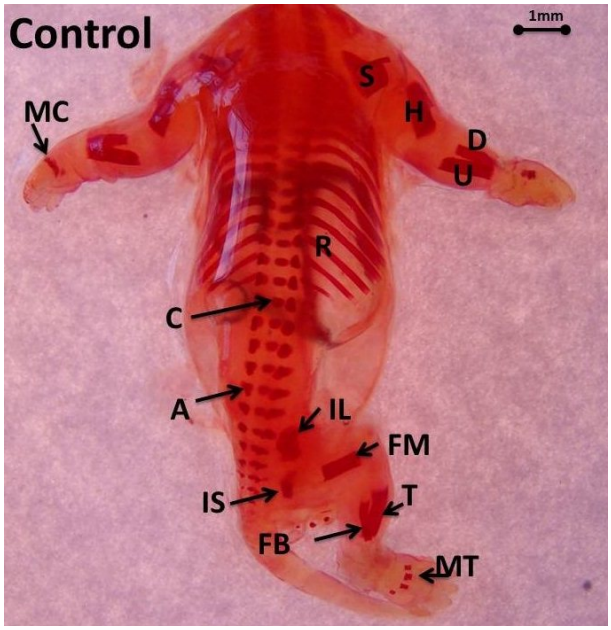


Fig. 5 (right). Posterior view of 20 day-old albino rat fetus from control group showing complete ossification of scapula (S), humerus (H), radius (D), ulna (U), metacarbal (MC), ribs (R), vertebral arches (A), centra (C), ilium (IL), ischium (IS), femur (FM), tibia (T), fibula (FB) and metatarsal (MT) ossification centers . x 15 (Alizarin red stain). Scale bar, 1 mm.

intra-gastric tube. They were weighted and observed daily throughout gestation for water intake and physical signs of toxicity following treatment.

Sample preparation

The pregnant rats were sacrificed at 9 o'clock on gestational day 20 by ether. The fetuses were obtained through caesarian section. The total number of implantation was determined by counting the metrial glands in uterine horns and the post-implantation loss or resorption was calculated. Each fetus was sacrificed by ether and totally eviscerated through a small midline incision in the anterior abdominal wall. Fetuses were skinned and rinsed in acetone for 1 day then 95 % ethyl alcohol for seven to ten days. The specimens were cleared in a solution of 1% KOH until the bones were clearly visible and stained with a fresh solution of alizarin red stain in 1% KOH. Finally the specimens were transferred to solutions containing 30%, 50% and 70% glycerin respectively and stored in pure glycerin (Dawson, 1926).

Skeletal stained fetuses were examined with the aid of a stereoscope microscope (Olympus SZ61; Olympus Corporation, Tokyo, Japan) for assessment of different bone ossification through scoring chart designed by (Nash and Persaud,

Table 4. Effects of prenatal administration of aluminum chloride and saffron on ossification of coxal, metacarpal and metatarsal bones in 20 day-old albino rat fetuses.

Bone ossification		Groups (no. of bones)		
		Control	$AlCl_3$	$AlCl_3$ + S
Ilium	**Complete**	120	84	100
	Partial	0	15 a, b	6 a
	Absent	0	5	2
Ischium	**Complete**	120	82	99
	Partial	0	17 a, b	7 a
	Absent	0	5 a	2
Pubis	**Complete**	120	80	99
	Partial	0	18 a, b	7 a
	Absent	0	6 a	2
Metacarpal bones	**Complete**	170	119	136
	Partial	0	21 a	15 a
	Absent	130	120	119
Metatarsal bones	**Complete**	168	107	137
	Partial	0	20 a	11 a
	Absent	132	133 a	122

Chi-square test: [a] P<0.05 compared to control, [b] P<0.05 compared to AlCl3 + S treated group.

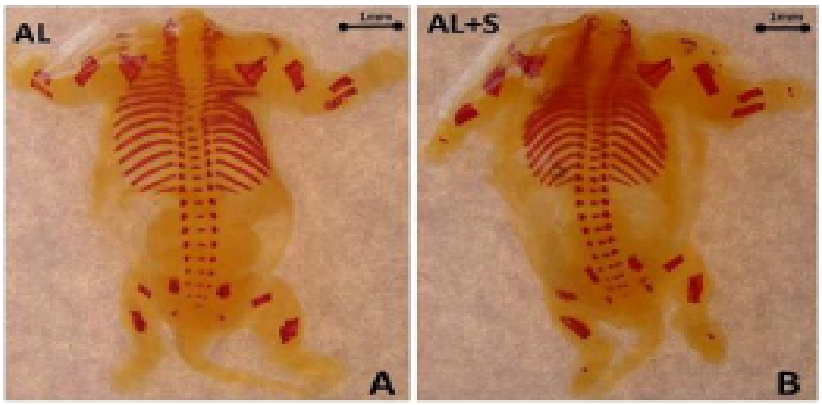


Fig. 6. Posterior views of 20 day albino rat fetuses from **(A) the** aluminum chloride treated group showing absent ossification of metacarpal, metatarsal, pubic, cervical and upper thoracic centra, last sacral arches and centra ossification centers and partial ossification of ischium, ilium, lumbar and upper sacral arches and centra. **(B)** Samples from the aluminum chloride + saffron treated group showing complete ossification of ischium, ilium, thoracic, lumbar and sacral arches and centra, 2 metatarsal and 2 metacarpal centers bilaterally and partial ossification of pubis. x 15 (Alizarin red stain). Scale bar ,1 mm.

1989). Ossification was scored as being complete ossification (CO), partial ossification (PO), or absent ossification (A). Delayed centers can be identified when the center was either lightly stained or asymmetrically developed (incomplete ossification). The length of the ossified parts of forelimb- (hummers, radius and ulna) and hindlimb- bones (femur, tibia and fibula) was measured through the eyepiece micrometer.

Statistical analysis

The chi-square and one-way analysis of variance (ANOVA) tests were used to statistically evaluate the results obtained from the control and treated groups.

RESULTS

It was observed that pregnant rats showed tremors, weakness, decrease water intake and drowsiness during the period of treatment in $AlCl_3$ treated group compared with other groups. Thesemanifestations were disappeared after stopping the treatment. $AlCl_3$ treated animals showed a weight loss during the period of treatment whereas control and $AlCl_3$ + S treated animals showed weight gain (fig. 1). Table 1 shows a significant increase in the number of resorption and dead fetuses in $AlCl_3$ group when compared with control and $AlCl_3$ + S treated groups.

The number of craniofacial bones that showed partial and absent ossification was significantly increased in $AlCl_3$ treated group compared with control and $AlCl_3$+S treated groups (table 2, figs. 2, 3 and 4). Regarding the ossification of vertebral bones, $AlCl_3$ treated group showed a significant increase in the number of partially ossified thoracic, lumbar and sacral arches and *centra* when compared to control and $AlCl_3$+ S treated groups. Also the number of absent ossification centers of vertebral arches, it was significantly increased in $AlCl_3$ treated group compared to other groups. $AlCl_3$ +S treated group showed a significant delay in the ossification of thoracic, lumbar and sacral vertebral arches and centra compared to control group (table 3, figs. 5 and 6).

It was noticed that mandible, ribs, forelimb (clavicle, scapula, humerus, radius and ulna) and hindlimb (femur, tibia and fibula) bones were completely ossified, whereas all carpal, tarsal bones and phalanges were absent in all examined fetuses in all groups. $AlCl_3$ and $AlCl_3$ + S treated groups showed a significant delay in the ossification of coxal (ilium, ischium and pubis), metacarpal and metatarsal bones compared to control group, but $AlCl_3$ +S treated group showed a significant improvement compared to $AlCl_3$ treated group (table 4, figs. 5, 7, 8).

Table 5 showed that there was a significant decrease in the length of the ossified part of the forelimb and hindlimb bones in $AlCl_3$ and $AlCl_3$ + S treated groups compared to control, but $AlCl_3$ + S treated group showed a significant increase in the length of the ossified part of forelimb and hindlimb bones compared to $AlCl_3$ treated group.

DISCUSSION

During the perinatal period, the mother is the prime source of aluminum exposure. Work in animal models (rats, mice, and rabbits) demonstrates that aluminum is distributed transplacentally and is present in milk (Golub and Domingo, 1996).

The findings of this study indicate that $AlCl_3$ treated animals showed a weight loss whereas control and $AlCl_3$ + S treated animals showed weight gain. Also a significant increase in the number of fetal resorption in $AlCl_3$ group when compared with both control and $AlCl_3$ + S treated groups were noticed, and the number of dead fetuses in $AlCl_3$ treated group was increased when compared with the control group. In previous studies using different Al-containing components, some controversial results were reported. Injection of Al lactate (15 to 40 mg/kg) in pregnant mice on different days of the gestation period had no effect on the weight of fetuses and did

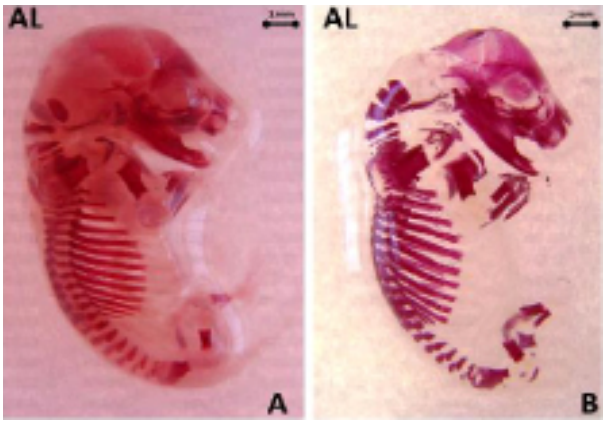


Fig. 7 (left). Lateral views of 20 day albino rat fetuses from aluminum chloride treated group showing: **(A)** partial ossification of metacarpal and metatarsal bones and absent ischial and pubic ossification centers; **(B)** partial ossification of ischium, pubis, two metacarpal and two metatarsal ossification centers. x 10 (Alizarin red stain). Scale bar, 1 mm.

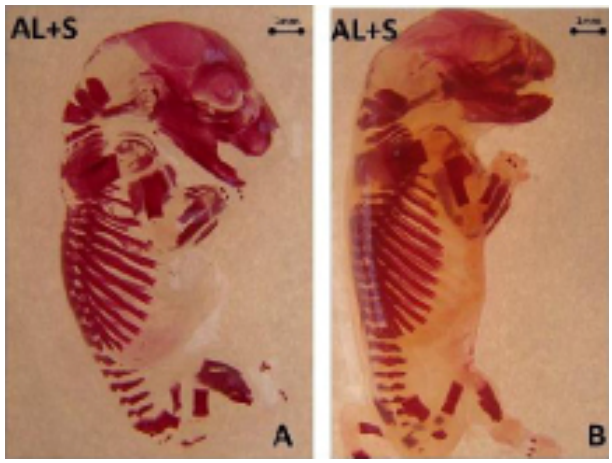


Fig. 8 (right). Lateral views of 20 day albino rat fetuses from $AlCl_3$ + S treated group showed: **(A)** complete ossification of 3 metacarpal and metatarsal ossification centers (bilaterally). **(B)** Complete ossification of 2 metacarpal, 3 metatarsal and coxal centers. x 10 (Alizarin red stain). Scale bar, 1mm.

not increase anomalies (Golub and Domingo, 1996). Also subcutaneous injection of aluminum on gestational days 7-15 does not have any effect on either the litter size or the body weight of pups in rats (Gonda and Lehotzky, 1996). And also aluminum lactate treatment did not have any effect on the weight gain of pups (Muller et al., 1990). On the other hand, some studies noticed low body weight after treatment of Al-containing components (Paternain et al., 1988; Colomina et al., 1998; Misawa and Shigeta, 1993). Lin et al., (1997) observed a dose-dependent relationship between aluminum intake and intrauterine growth retardation in mice, and suggested that excessive aluminum ingestion during pregnancy may be a contributing factor in perinatal deaths.

The present study revealed that the number of craniofacial bones that showed partial and absent ossification was significantly increased in $AlCl_3$ treated group compared with control and $AlCl_3$+S treated groups. Regarding the ossification of vertebral bones, $AlCl_3$ treated group showed a significant increase in the number of partially ossified thoracic, lumbar and sacral arches and *centra* when compared to control and $AlCl_3$+ S treated groups. Also the number of absent ossification centers of vertebral arches was significantly increased in $AlCl_3$ treated group compared to other groups. $AlCl_3$ +S treated group showed a significant delay in the ossification of thoracic, lumbar and sacral vertebral arches and *centra* compared to control group. $AlCl_3$ and $AlCl_3$ + S treated groups showed a significant delay in the ossification of coxal (ilium, ischium and pubis), metacarpal and metatarsal bones compared to control group, but $AlCl_3$ +S treated group showed a significant improvement compared to $AlCl_3$ treated group. There was also a significant decrease in the length of the ossified part of the forelimb and hind-limb bones in $AlCl_3$ and $AlCl_3$ + S treated groups compared to control, but $AlCl_3$ + S treated

Table 5. Effects of prenatal administration of aluminum chloride and saffron on the length of the ossified part of albino rat fetuse forelimb and hind-limb bones.

Bones	Groups (Length of bone (cm) Mean ± SD)		
	Control	$AlCl_3$	$AlCl_3$ +S
Humerus	0.23±0.013	0.18±0.008a, b	0.215±0.012a
Radius	0.17±0.015	0.13±0.017a, b	0.16±0.017
Ulna	0.22±0.012	0.175±0.008a, b	0.20±0.014a
Femur	0.14±0.009	0.08±0.006a, b	0.12±0.019a
Tibia	0.19±0.013	0.15±0.01a, b	0.175±0.008 a
Fibula	0.17±0.009	0.13±0.012a, b	0.155±0.012a

ANOVA test: [a] P<0.05 compared to controls. [b] P<0.05 compared to AlCl3 +S treated group.

group showed a significant increase in the length of the ossified part of forelimb and hind-limb bones compared to $AlCl_3$ treated group.

This result was in agreement with another study which reported that Al nitrate (13 to 62 mg/kg) caused increased anomalies of external structures and the skeletal system in rat fetuses (Paternain et al., 1988). In another study that administered 100 to 200 mg/kg of AlCl3 in rats on days 14 to 18 of gestation caused growth retardation, increase of atrophy and fetal death, as well as skeletal anomalies (Benett et al., 1975). Exposure of rats to aluminum nitrate by gavage from the 6^{th} to 14^{th} day also resulted in increased the incidence and types of external, visceral, and skeletal malformations and variations in all the treated groups (Paternain et al., 1988). Oral aluminum administration during pregnancy, at doses that also lead to reduced maternal weight gain, produces a syndrome including growth retardation, delayed ossification, and malformations in perinates (Golub and Domingo, 1996). In the Al-treated groups, the resorption rates/fetus were 25.5%, 21.2%, and 23.3%, respectively, while no resorption in the control groups was noted. External skeletal deformities were seen most commonly in the lower and upper limbs, spinal column, and skull in the fetuses of the experimental groups. In addition, the anomaly rate increases considerably with fetuses that were exposed earlier in gestation (Abbasali et al., 2005). Mestaghanmi et al., (2002) observed that $AlCl_3$ changed plasma and tissue iron, phosphorus, magnesium, zinc and calcium concentrations of pregnant rats and their offspring at the highest dose used. These disturbances in essential elements induced disturbances on the skeleton and neurological functions. It inhibits the action on Ca++ ATPase in brain cells in rats (Jagannath, 1992).

Controversy results were reported after studying the effects of AlCl3 (75 mg/kg) on days 6 to 15 of gestation on the embryos/fetuses of mice and noticed that the number of external skeletal anomalies did not increase (Colomina et al., 1998).

These skeletal changes in Al3 group may be due to the fact that Aluminum ion (Al3+) is a trivalent cation, and has a high affinity for negatively charged groups. It has been proposed that Al preferentially interacts with phosphate groups, such as nucleic acids and phosphorylated proteins. In this way, Al remarkably decreases DNA and RNA synthesis13, 19 and inhibits embryonic cell proliferation and protein synthesis. This mechanism can explain the toxic effects of Al on the embryo and fetus. There are also other cellular mechanisms by which Al is thought to exert its toxicity, including increasing the blood-brain barrier permeability, interference with phosphorylation - and de-phosphorylation reactions, altered iron metabolism with subsequent free-radical production, and disruption of second messenger systems (Agrawal et al., 1996).

The results conform to a variety of studies in which saffron, crocin and safranal had protective effects against oxidation-induced tissue injuries due to their antioxidant properties.

The results obtained in the present investigation suggest that the saffron extract and its active constituents, crocin and safranal, have an overall protective effect against Al3 treated group.

The improvement in groups which administrate the saffron may be due to the fact that saffron extract is chemo-preventive and showed protective effects on genotoxins- induced oxidative stress in Swiss albino mice (Premkumar et al., 2003). Administration of aqueous green coffee and saffron extracts can improve the hazards of $AlCl_3$, and also can modulate the antioxidant system and oxidative stress in balance (Fahmy et al., 2011).

The aqueous extract of safflower seed significantly accelerated rates of osteoblast differentiation in the experimental group as compared to the control group in murine osteoblastic cells of the MC3T3-E1 line cultured in modified Eagle's minimum essential medium (Jang et al., 2007). Phytoestrogen rich safflower seeds demonstrated a protective effect on bone loss caused by estrogen deficiency, without substantial effect on the uterus examined by scanning electron microscopy and histomorphometric analysis. The beneficial effect of safflower seeds may be mediated, at least in part, by the stimulating effect of polyphenolic compounds on proliferation of osteoblasts (Kim et al., 2002). It was reported that crocin and dimethyl-crocetin isolated from saffron were non-mutagenic (Salomi, 1991). It has been reported that saffron or the compounds it contains, such as crocin and dimethylcrocin, are not mutagenic or genotoxic (Salomi et al., 1991). Premkumar et al., 2001; 2003; and 2006) also showed that a saffron aqueous extract protects from genotoxicity as well as genotoxins-induced oxidative stress in mice. It was suggested that saffron could exert its antigenotoxic and chemopreventive effects by the modulation of antioxidants and/or detoxification systems (Premkumar et al., 2001, 2006).

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