ejpmr, 2020,7(4), 591-600

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EFFECT OF SELENIUM AGAINST NALUFIN INDUCED MORPHOLOGICAL AND ENDO-SKELETAL ABNORMALITIES IN CHICK EMBRYOS

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Article Received on 14/02/2020 **Article Revised on 05/03/2020** **Article Accepted on 26/03/2020**

ABSTRACT

Opioids administration during pregnancy can affect the embryonic development and can lead to different malformations in the developing embryo such as growth retardation, spontaneous abortion, skeletal defects and many limb deformities. The present study aimed to determinate the possible hazard effects of the new generation opioid pain killer nalufin on the morphology and endo-skeletal system of the developing chick embryo. Another aim was to investigate the ameliorative role of selenium on these effects. The fertilized eggs of leghorn chickens (*Gallus gallus domesticus*) were *in ovo* injected with 0.2ml of either nalufin (20mg/egg, single dose) or selenium (0.1 mg/kg, single dose) or combined injection. The injection of nalufin resulted in increased mortality rate and reduction in lengths and weights of embryos with many malformations including omphalocele, limb deformities, head enlargement, scanty feather, exencephaly, short beak and subcutaneous hemorrhage. Furthermore, nalufin led to many endo-skeletal malformations in the skull, vertebral column, ribs, pubis, limbs, incomplete ossifications of the bones and decreased length of the long bones. The co-injection of selenium ameliorated the mentioned malformations induced by nalufin.

KEYWORDS: Chick embryo; Nalufin; Opioids; Selenium; Endoskeleton; Teratogenicity; Morphology; Embryology.

INTRODUCTION

Opioid analgesics are considered as the most commonly and effective pharmacologic agents used for the management and treatment of moderate to severe pain.[1,2] Mothers are one of the most important affected analgesic consumers that addiction side-effects will involve them and their next generation.[3] Opioid analgesics are also often used during obstetric labor; however, because opioids cross the placental barrier easily and reach the fetus, care and caution must be implemented to minimize the incidence of neonatal depression.[4] Opioid use during pregnancy can be associated with negative pregnancy and infant outcomes, low birth weight, increased risk of spontaneous abortion, preterm birth, decreased head circumference, many limb deformities, sudden infant death and infant neurobehavioural abnormalities.[5,6,7] In addition, using opioids lead to increased risk of skeletal system damage affecting bone and resulting in a reduction in bone mineral density and induces many skull deformities such as exencephaly and cranioschisis.[8,9]

Opioids are classified into old generation such as morphine and methadone and new generation pain killers such as nalufin. Many studies confirmed the teratogenic effects of old generation pain killers on human, rat and chick embryos.[10] On the other hand, there are no sufficient studies to investigate the teratogenicity of newer opioids including nalufin.[11]

Nalufin or (Nalbuphine) is a semi-synthetic narcotic that is equipotent to morphine in clinical use.[12] It is usually used for obstetrical analgesia during labor and delivery and also used to decrease tumor related pain.[13] Based on previous studies oral administration of morphine as a pain killer can pass the placental barrier and have destructive effects on the developing embryo.[14]

Selenium is an essential trace element with many beneficial roles in biochemical and physiological processes as well as it is a powerful antioxidant enzyme.[15,16,17] It is found in food nutrients in different forms including organic selenium compounds such as selenomethionine (SeMet, mainly cereals and Se yeast)

and selenocysteine (SeCys, animal food) and inorganic selenium such as selenite that is added to dietary supplements with accepted maximal total content of 0.5 mg Se/kg.[18,19] Selenium was reported to improve the body weight in silver treated rats[20] and in Japanese quail and chickens when used as a dietary supplement.[21] Turan *et al*.[22] found that the use of selenium in combination with vitamin E and C improved the bone tissue and mineral density and decreased the heparin induced osteoporosis in rabbit bones.

Therefore, the present study aimed to investigate the possible teratogenic effects of the new generation pain killer nalufin on the developing chick embryo. Meanwhile, the possible ameliorative role of selenium against these effects was parallelly investigated in terms of morphological and endo-skeletal parameters.

MATERIALS AND METHODS

Egg incubation and grouping

Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by Faculty of Science, Menoufia University, Egypt (Approval No. MNSE2187) and according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Normal fertilized eggs of leghorn chickens (*Gallus gallus domesticus*) were obtained from a hatchery at Elshohda, Menoufia governorate. Before incubation at 37°C in an artificial incubator, eggs were cleaned with distilled water followed by 70% ethanol weighed (50 ± 5 g), then the upper most spot of the egg shell was marked with a permanent marker. To ensure the relevant humidity (65%), an open 1-liter container filled with distilled water was placed at the bottom of the incubator. The eggs were put horizontally on metal racks and turned over, at least three times a day. On the fifth day, eggs were candled and the unfertilized eggs were excluded from the experiment and the remaining eggs were divided into five groups 10 eggs each.

1- Group A was not subjected to any injection (Control group).
2- Group B was injected *in ovo* with 0.2ml of distilled water (Sham group).
3- Group C was injected *in ovo* with 0.2ml Nalufin at a dose of 20mg/egg.[23]
4- Group D was injected *in ovo* with 0.2 ml selenium at dose of 0.1 mg/kg.[24]
5- Group E was injected *in ovo* with 0.2 ml of 1:1 mixture of nalufin (20mg/egg) and selenium (0.1 mg/kg).

Nalufin ampules obtained from (Spimaco Misr pharma company, Egypt). Selenium was obtained in a pure powder form 98% (Lobal chemie company, India) and dissolved in sterile distilled water.

At the sixth day of incubation, a tiny hole was pierced into the lateral edge of the egg at the region of the air sac with a sharp and thin needle under septic conditions. Using a sterile syringe, 0.2 ml of fluid was directly injected into the air sac. The hole was carefully sealed with molten paraffin wax after the single dose injection in all experimental groups. The egg was returned to the incubator after injection to allow the embryonic development.

Embryo collection

All eggs were opened on the 20th day of incubation. The egg shells were broken with a scalpel and the embryos were carefully freed from the egg shell. The crown-rump length, body weight and morphological abnormalities were recorded and representatives from all groups were photographed. The embryos were fixed in 10% neutral buffered formalin for endo-skeletal preparation.

Investigated parameters

Morphometric parameters

The crown-rump length (cm) and body weight (gm) were recorded.

Endo-skeletal investigation

For endo-skeletal preparation, double staining transparency technique was applied using the chondrogenic indicator Alcain blue and osteogenic indicator Alzarin red S for staining cartilage and bone, respectively. This has been achieved following a method originally introduced by Cortés-Delgado *et al*.[25] and modified by Badawy *et al*.[26] Photographs of representative samples were taken using Sony digital camera. Lengths of long bones *i.e* Humerus, radius, ulna, femur, tibia and fibula were measured.

Data evaluation and statistical analysis

All data sets were expressed as mean ± standard error of the mean (SEM). The data were analyzed statistically for normal distribution (student's T test) and homogeneity of variances (Levene test) Independent-samples T test using statistical package for the social sciences (IBM SPSS) statistics software for Windows, Version 22 (IBM Corp, Armonk, NY, USA). Differences were considered insignificant whenever $P>0.05$. The significances of the obtained data were classified into two categories, i.e. $P<0.0001$ and $P<0.05$ according to the obtained P values.

RESULTS

Mortality rate

The percentage of mortality rate was recorded at the end of experimentation, i.e. 20 days of incubation by calculating the number of dead embryos that died normally or as a result of nalufin administration. The number and percentage of both living and dead embryos were recorded (Table 1). The results showed that mortality in control, sham and selenium groups was low (20% per each group). On the other hand, the mortality rate of embryos *in ovo* injected by nalufin was high compared with control (47.6%). The injection of selenium in combination with nalufin decreased the mortality rate (23.5%).

Table (1): Percentage of mortality rate (%) recorded at the end of experimentation in different groups.

Groups	No. eggs	No. live embryos	No. dead embryos	Mortality rate (%)
Control	**40**	**32**	**8**	**20%**
Sham	**40**	**32**	**8**	**20%**
Se	**40**	**32**	**8**	**20%**
Nalufin	**63**	**33**	**30**	**47.6%**
Nalufin + Se	**34**	**26**	**8**	**23.5%**
The percentage was calculated individually according to the original number of each group.				

Morphological and morphometric investigations

The effect of injection of nalufin and selenium, either individually or in combination, on chick embryos during organogenesis, i.e. sixth day of incubation compared with the control group can be summarized as follows.

Morphological abnormalities

All embryos of control, sham and selenium injected groups showed normal structure (Fig. 1A-C), although there was low incidence of omphalocele, limb deformities and head enlargement (18.75%, 12.5% and 12.5% for the three groups, respectively) (Table 2).

On the other hand, chick embryos injected with nalufin showed a variety of malformations (Fig. 1 D&E; Table 2) including growth retardation and high incidence of omphalocele (75.75%; Fig. 1 D&E), head enlargement and limb deformities which included bifurcation of first toe, clinodactyly and flexed limbs (45.5%; Fig. 1D). Other malformations included scanty feather (18.2%; Fig. 1E), short beak and exencephaly (18.2%; Fig. 1D). The least morphological abnormality was subcutaneous hemorrhage with only 9.1% of embryos showing this defect. Co-injection of selenium with nalufin resulted in amelioration in the morphology of chick embryos as well as decrease in the presence of omphalocele to 30.77%, limb deformities and head enlargement to 15.38% (Fig. 1F)

Table (2): percentage of morphological abnormalities (%) recorded at the end of experimentation, *i.e*. the 20th day of incubation in different groups.

Malformation	Groups				
	Control n=(32)	**Sham n=(32)**	**Se n=(32)**	**Nalufin n=(33)**	**Nalufin +Se n=(26)**
Omphalocele	18.75% (6)	18.75% (6)	18.75% (6)	75.75% (25)	30.77% (8)
Limb deformities	12.5% (4)	12.5% (4)	12.5% (4)	45.5% (15)	15.38 % (4)
Head enlargement	12.5% (4)	12.5% (4)	12.5% (4)	45.5% (15)	15.38% (4)
Scanty feather	0%	0%	0%	18.2% (6)	0%
Exencephaly	0%	0%	0%	18.2% (6)	0%
Short beak	0%	0%	0%	18.2% (6)	0%
Subcutaneous Hemorrhage	0%	0%	0%	9.1% (3)	0%
The percentage of every abnormality was calculated individually according to the original number of each group.					

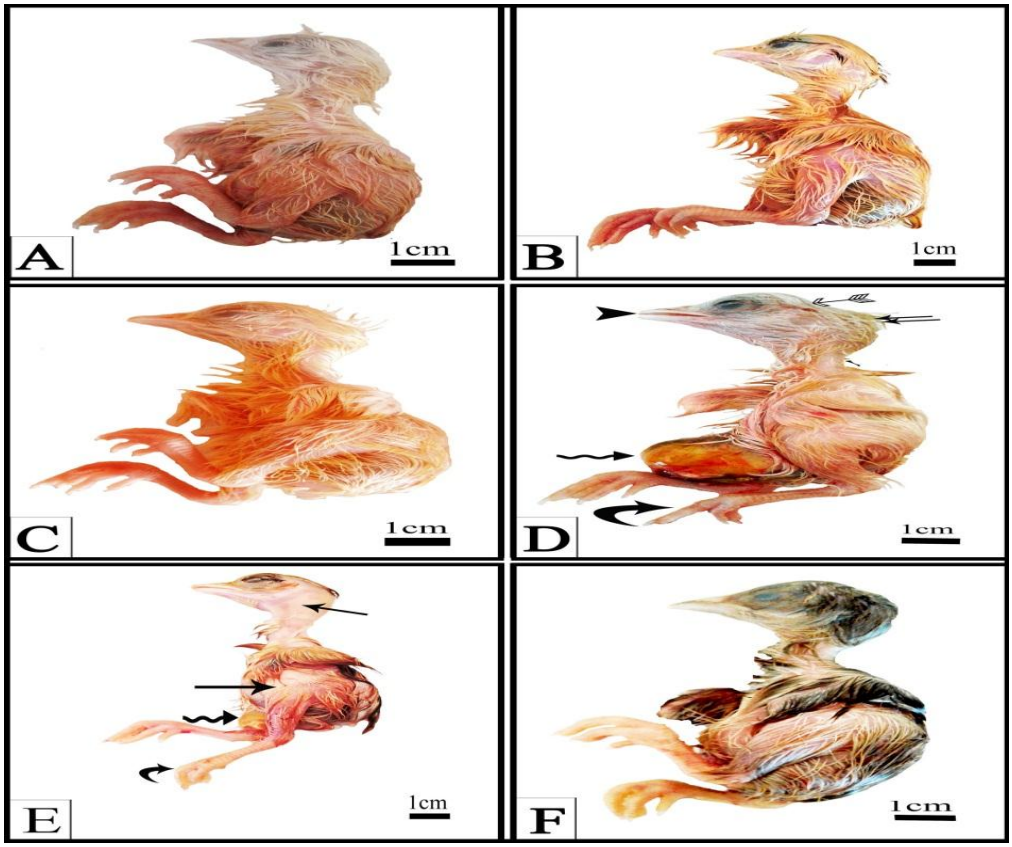

Figure (1): Photographs of embryos aged 20 days of incubation from control (A), sham (B), selenium (C), nalufin (D&E) and nalufin+Selenium (F) groups. A,B& C showing almost normal morphology. D showing short beak (head arrow), exencephaly (tailed arrow), head enlargement (double arrow), omphalocele (wavy arrow) and flexed limb (curved arrow). E showing growth retardation, scanty feather (arrow), clinodactyly (curved arrow) and omphalocele (wavy arrow). F showing marked improvement in the shape and length compared with the nalufin group. Scale bar = 1cm

Morphometric analysis

The chick embryo-toxicity data from the control, sham, selenium, nalufin and nalufin + selenium injected groups were presented in Table (3). The results showed that nalufin led to evident growth retardation compared with the control group as it affected the crown-rump length and body weight. On the other hand, there was a marked amelioration in different growth parameters in embryos injected with selenium either individually or in combination with nalufin.

Crown-rump length

Table (3), showed that chick embryos of control, sham and selenium injected groups had approximately similar values for the crown-rump length (9.916 ±0.030; 9.900 ±0.0365; 9.850 ±0.067 for the three groups, respectively). On the other hand, the length of embryos injected with nalufin showed a highly significant decrease in the crown-rump length compared with control group (7.650±0.056; 9.916 ±0.030 for the two groups, respectively). Meanwhile, embryos injected with nalufin and selenium displayed a low significant increase in the length compared with nalufin alone (8.980 ±0.090; 7.650±0.056 for nalufin + selenium and nalufin groups respectively) and led to low significant decrease compared with control groups.

Body weight

Table (3), illustrated differences in the body weight of the chick embryos aged 20 days in different groups. Embryos from control, sham and selenium groups had very close values (44.116±0.492; 43.983±0.286; 43.683±0.190 for the three groups, respectively). On the other hand, the body weight of embryos injected with nalufin showed a highly significant decrease in the body weight compared with control group (28.033±0.276; 44.116±0.492 for the two groups, respectively). However, embryos injected with nalufin and selenium displayed marked improvements of body weight compared with nalufin alone (43.000±0.447; 28.033±0.276 for nalufin + selenium and nalufin groups respectively). This significant amelioration of body weight led to insignificant difference towards the control groups.

Table (3): Crown-rump length and body weight of chick embryos aged 20 days of incubation in different groups.

Groups	Fetal Growth Parameters	
	Length	Weight
Control	9.916 ±0.030	44.116±0.492
Sham	9.900 ±0.0365	43.983±0.286
Se	9.850 ±0.067	43.683±0.190
Nalufin	7.650±0.056**	28.033±0.276**
Nalufin + Se	8.980 ±0.090*a	43.000±0.447b

Data are represented as mean ± SEM.
Asterisks (**) and (*) refer to the P values compared with the control group.
(**) P< 0.0001 (*) P< 0.05
b= highly significant (p< 0.0001) compared with nalufin group.
a= low significant (P< 0.05) compared with nalufin group.
Endo-skeletal investigation
Skeletal abnormalities

Control group
Examining the double stained endo-skeletal system of 20-day old embryos revealed that most parts of the skull exhibited a large degree of ossification. However, the squamosal, interorbital, otic and nasal capsules were less ossified. The bones of the upper jaw were slightly longer than those of the lower jaw. The vertebral column of the control group had a normal structure without any lateral flexion. The sternum was enlarged forming the carina and it was completely cartilaginous. The ischium and ilium were completely ossified and stained heavy red, but the pubis was completely cartilaginous in nature, except in its middle part that was ossified and slightly curved. The long bones including humerus, radius and ulna, femur and tibiotarsus, carpometacarpus and tarsometatarsus were completely ossified except in the epiphyseal regions. Most of limb phalanges were ossified (Fig. 2A).

Sham and selenium groups
The endo-skeletal structure of the embryos from sham and selenium groups had no significant differences compared with the control group (Table 4; Fig. 2B&C).

Nalufin group
The chick embryos from nalufin group showed many skeletal abnormalities. The latter in the axial skeleton included the skull cap, vertebrae, sternum, and ribs. Bones of the embryos from this group showed delayed or incomplete ossification (Table 4; Fig. 2D-H).

Defects of the skull and beak constituted 33.3% of the whole abnormalities (Table 4). The skull malformation included lost parts of its bones especially frontal and parietal, short upper and lower jaws (Fig. 2D,G&H).

All embryos suffered from malformation in the vertebral column 100% (Table 4). Cervical scoliosis (33.3%; Fig. 2E&G). Kinked tail and pygostyle (73.3%) as well as caudal regression syndrome (20%; Fig. 2D-F).

Malformation in the ribs were represented in fifth (20%, Table 4) of the embryos and appeared in the form of incomplete ossification. About 73.3% of embryos had incomplete ossification and curved pubis (Table 4, Fig. D&H). Also, the hind limbs malformation included clinodactyly and furcation of the first toe with percentage of (46.7%; Fig. 2G).

Nalufin and selenium
The co-injection of selenium with nalufin *in ovo* decreased the endo-skeletal malformations of embryos compared with nalufin alone. For instance, there were no abnormalities in the skull region, the vertebral column abnormalities were found in only 16.7% of the embryos and were in the form of incomplete ossification of caudal vertebrae. Moreover, the incomplete ossification of pubis and its curvation decreased to 16.7%. The defects of hind limb also decreased to 16.7% (Table 4; Fig. 2I). The ribs showed complete ossification.

Table (4): Endo-skeletal abnormalities of chick embryos aged 20 days in different groups (percentage %).

Endo-skeletal abnormality	Groups				
	Control n=(12)	Sham n=(12)	Se n(12)	Nalufin n=(30)	Nalufin + Se n=(12)
Skull	0%	0%	0%	33.3% (10)	0%
Vertebral column	0%	0%	0%	100% (30)	16.7%(2)
Cervical kyphosis	0%	0%	0%	33.3% (10)	0%
Scoliosis	0%	0%	0%	33.3%(10)	0%
Caudal regression syndrome	0%	0%	0%	20%(6)	0%
Kinked tail and pygostyle	0%	0%	0%	73.3(22)	0%
Incomplete ossification of tail	0%	0%	0%	73.3%(22)	16.7%(2)
Incomplete ossification of ribs	0%	0%	0%	20%(6)	0%

Pubis	0%	0%	0%	73.3%(22)	16.7%(2)
Hind limb malformations	0%	0%	0%	46.7%(14)	16.7%(2)
The percentage of every abnormality was calculated individually according to the original number of each group.					

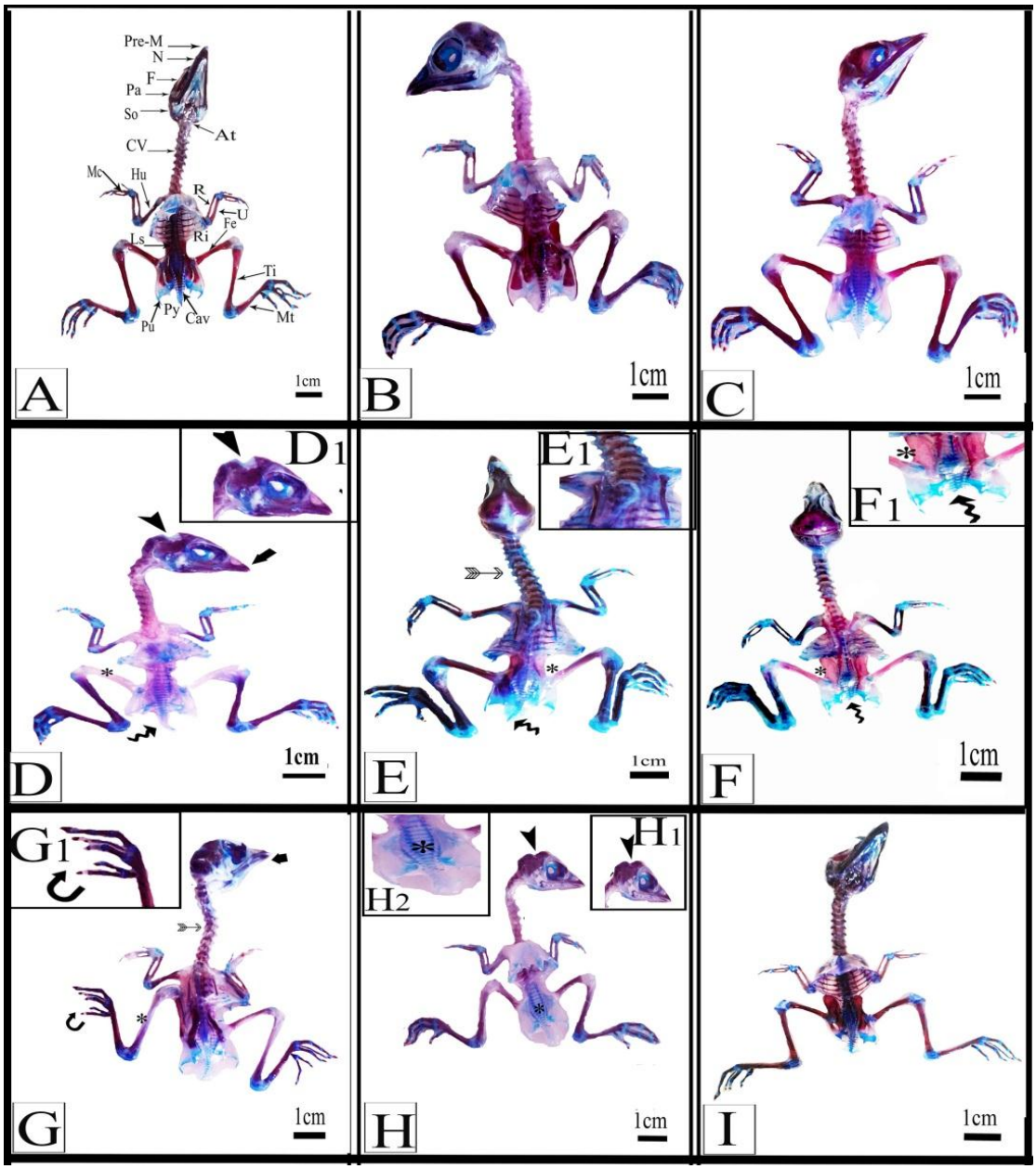

Figure (2): Photographs of different views of the endo-skeletal system of chick embryos aged 20 days from control (A), sham (B), selenium (C), Nalufin (D-H) and Nalufin+selenium (I). (A, B & C) showed well-formed endo-skeletal with normal ossification. Thick arrow for short jaws (D, G), head arrow for loss parts of the skull cap (D, H), wavy arrow for kinked tail and pygostyle (D-F), tailed arrow for scoliosis (E, G), curved arrow for first toe bifurcation (G), star (*) for incomplete ossification (D-H). (I) showed amelioration in the endo-skeletal system and ossification pattern. Scale bar = 1cm.

Length of ossified centers

Measurements of the pattern of ossification centers of long bones showed variations in their lengths between different groups (Table 5). The length of humerus in control, sham and selenium groups was quite similar (1.150±0.048cm, 1.133±0.055cm and 1.100±0.025cm, for the three groups respectively). On the other hand, length of humerus decreased in embryos in nalufin group compared with control group (0.725±0.011cm). Co-injection of selenium with nalufin increased the length of humerus compared with nalufin group (0.890±0.042cm). The length of radius in control, sham and selenium group showed insignificant differences (0.938±0.004cm, 0.931±0.006cm and 0.923±0.007cm, for the three groups

respectively). Contrarily, the length of radius decreased in embryos in nalufin group compared with control group (0.603±0.003cm). Meanwhile, co-injection of selenium with nalufin significantly increased the length of radius compared with nalufin group (0.810±0.008cm). The difference in the length of ulna in control, sham and selenium group were insignificant (0.935±0.008cm, 0.925±0.007cm and 0.918±0.006cm, for the three groups, respectively). However, the length of ulna showed a highly significant decrease in embryos of nalufin group compared with control (0.591±0.010cm). Co-injection of selenium with nalufin resulted in insignificant increase in the length of ulna compared with nalufin group (0.800±0.004cm). There was no significant change in the length of carpo-metacarpus in control, sham and selenium groups (0.813±0.003cm, 0.808±0.003cm and 0.805±0.002cm, for the three groups respectively). On the other hand, the length of carpo-metacarpus decreased significantly in embryos in nalufin group compared with control group (0.413±0.002cm). Co-injection of selenium with nalufin significantly ameliorated the length of carpo-metacarpus compared with nalufin group (0.801±0.002cm).

The length of femur in control, sham and selenium groups showed insignificant differences (1.833±0.007cm, 1.825±0.007cm and 1.823±0.006cm, in the three groups respectively). There was a highly significant decrease in the length of femur in embryos injected with nalufin group compared with control (1.300±0.036cm). Co-injection of selenium with nalufin increased the length of femur significantly compared with nalufin group (1.811±0.008cm). The same results were found in the length of tibiotarsus between the control, sham and selenium groups as there were insignificant difference (2.525±0.008cm, 2.516±0.007cm and 2.515±0.006cm, for the three groups respectively). On the other hand, the length of tibiotarsus decreased in nalufin group compared with control (1.320±0.03cm). Co-injection of selenium with nalufin resulted in significant difference in the length of tibiotarsus compared with control group and highly significant increase compared with nalufin group (2.300±0.005cm). Finally, the length of tarso-metatarsus in control, sham and selenium group also had insignificant differences (1.725±0.008cm, 1.723±0.007cm and 1.721±0.006cm respectively). On the other hand, length of tarso-metatarsus decreased in embryos from nalufin group compared with control (1.301±0.037cm). There was insignificant difference in the length of tarso-metatarsus in embryos injected with selenium and nalufin compared with control group and highly significant increase compared with nalufin group (1.700±0.005cm).

Table (5): Effect of nalufin injection on the length of ossified centers of long bones in 20 days chick embryos in different groups.

Groups	Humerus	Radius	Ulna	Carpo-metacarpus	Femur	Tibiotarsus	Tarso-metatarsus
Control	1.150±0.048	0.938±0.004	0.935±0.008	0.813±0.003	1.833±0.007	2.525±0.008	1.725±0.008
Sham	1.133±0.055	0.931±0.006	0.925±0.007	0.808±0.003	1.825±0.007	2.516±0.007	1.723±0.007
Se	1.100±0.025	0.923±0.007	0.918±0.006	0.805±0.002	1.823±0.006	2.515±0.006	1.721±0.006
Nalufin	0.725±0.011**	0.603±0.003**	0.591±0.010**	0.413±0.002**	1.300±0.036**	1.320±0.037**	1.301±0.037**
Nalufin + Se	0.890±0.042*a	0.810±0.008*a	0.800±0.004*a	0.801±0.002b	1.811±0.008b	2.300±0.005b	1.700±0.005b

Data are represented as mean (cm) ± SEM.
Asterisks (**) and (*) refer to the P values compared with the control group.
(**) P< 0.0001 (*) P< 0.05
b= highly significant (p< 0.0001) compared with nalufin group.
a= low significant (P< 0.05) compared with nalufin group.

DISCUSSION

The present study showed that injection of nalufin during the organogenesis phase of chick embryos increased the mortality rate compared with control and sham groups. These results are in agreement with Nasiraei-Moghadam *et al*.[27] who reported that morphine with a dose of 0.01mg/ml led to high mortality rate in rat embryos and also in agreement with Salahshoor *et al*.[28] who found that injection of 20 mg/kg of morphine increased the mortality rate in the same experimental model.

In the current study, injection of nalufin led to various morphological abnormalities in chick embryos such as increased percentage of omphalocele, exencephaly, limb deformities, scanty feather, head enlargement and short beak. This is in agreement with Källén *et al*.[29] and Lind *et al*.[6] who found that using opioids during pregnancy lead to similar congenital malformations in embryos due to the metabolism of this opioids in the gastrointestinal tract. Jurand[8] also found that injection of methadone hydrochloride at a dose of 20 or 40 mg per kg body weight resulted in malformation in mouse, rabbit and chick embryos such as excencephaly which was suggested to occur due to extensive dilatation of blood vessels in the averted brain hemispheres, most probably due to an abnormal angiogenesis which plays an important causative role in the pathogenesis of anencephaly due to the failure of integration of primordial cerebral vessels into the systemic circulation.

According to this study, injection of nalufin led to significant growth retardation proved by the decreased crown rump length and weight of the developing chick embryos. Similarly, several researches reported that

morphine injection at different doses in either pregnant rats or mice resulted in decreased length and weight of the fetuses.[30-33] This growth retardation is suggested to be due to the fact that morphine increases dopamine and xanthine oxidation and consequently, can increase ROS.[34] Morphine has also been reported to metabolize free radicals and this generation of free radicals in turn leads to general loss of body mass.[28]

Various malformations in the endo-skeletal system of chick embryos have been induced due to nalufin injection in the current study, in addition the length of the ossified parts of different long bones was significantly decreased. Jurand[8] found that methadone hydrochloride injection led to loss parts of the skull and Z-shaped kinkage of the spinal cord leading to curvation in vertebral column and the fetuses had rib and vertebral fusions. Similarly, Shadid and Barrett[35] found that morphine administration led to osteoporosis and decreased ossification of bones. Also, Ezzatabadipour *et al*.[36] and Janas and Folwarczna[9] reported that injection of morphine at different doses in male rats led to decreased length of long bones in a dose dependent manner. These endo-skeletal malformations could be attributed to the fact that opioids have receptors on bones, thus affect the osteoblasts and bone remodeling.[37] In addition, the oxidative stress effect of morphine may reduce cell numbers in the proliferative zone of the bone.[36] Moreover, it has been found that mu, delta and kappa receptors on osteoblast-like cells (MG-63) and the high concentration of morphine, as an agonist of mu receptor sites, prevented the synthesis of osteocalcin, which is a marker of osteoblastic activity.[38]

The present study showed that co-injection of selenium as antioxidant compound with nalufin led to improvements in the percentage of mortality, length and weights of chick embryos compared with nalufin group. These results are in agreement with Li *et al*.[39] who found that addition of selenium to the diet of cocks at a dose 10 mg/kg of diet improved the length and weight of embryos that decreased by cadmium uptake. Also, Surai and Fisinin[40] found that addition of selenium in maternal nutrition of chickens improved the reproduction and decreased the mortality rate. Also, it was proved that adding a high level of selenium in the diet of laying hens significantly increased egg production and resulted in selenium enriched eggs.[41] The ameliorating effects of selenium could be attributed to its ability to remove ROS from cell membrane and increase cell number and consequently increase the general body mass.

This study showed that co-injection of selenium with nalufin improved the length and ossification of bones. This is in agreement with Zofkova *et al*.[42] who found that selenium is very important in increasing bone mass and bone quality. This is due to its effect as an antioxidant that prevented ROS production by osteoclasts during bone remodeling.[43]

CONCLUSION

The present study showed that nalufin injection may have deleterious effects on the morphology and endoskeleton of the developing embryos and its usage should be restricted to high necessity during pregnancy. On the other hand, the current study showed that selenium has an important ameliorating effects against the teratogenic effects induced by nalufin. Though, further studies are needed to understand the actual mechanisms of nalufin teratogenicity and selenium ameliorating effect.

The authors declared that they have no conflict of interest.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Abbreviations: Caudal vertebrae (Cav); Cervical vertebrae (CV); Femur (Fe); Frontal (F); Humerus (Hu); Nasal (N); Metacarpus (Mc); Metatarsus (Mt); Parietal (Pa); Pre-maxilla (Pre-M); Pubis (Pu); Pygostyle (Py); Radius (R); Reactive Oxygen Species (ROS); Ribs (R); Squamosal (So); Tibia (Ti); Ulna (U).

REFERENCES

1. Bannister K. Opioid-induced hyperalgesia: where are we now? Curr Opin Support Palliat Care, 2015; 9: 116-21. DOI:10.1097/SPC.0000000000000 137.
2. Tsai L, Doan T. Breastfeeding among mothers on opioid maintenance treatment: a literature review. J Hum Lact, 2016; 32: 521-9.
3. Malhotra S, Khanna S. Safety of analgesics in pregnancy. IJOGR, 2016; 3: 208-12.
4. Klaman S, Isacs K, Lcopoid A, Perpich J, Hayashi S, Vender J, Campopiano M, Jones H. Treating women who are pregnant and Parenting for opioid use disorder and the concurrent care of their infants and children: literature review to support national guidance. J Add Med, 2017; 11: 178-90. DOI:10.1097/ADM.000000 0000000308
5. Meyer M, Johnston A, Crocker A, Heil S. Methadone and buprenorphine for opioid dependence during pregnancy: A retrospective cohort study. J Add Med, 2015; 9: 81-6. DOI: 10.1097/ADM.0000000000 000092.
6. Lind J, Julia D, Ailes E, Gilboa S, Frey M, Honein M, Dowling N, Dawson A, Razzaghi H, Creanga A, Broussard C. Maternal use of opioids during pregnancy and congenital malformations: a systematic review. J Pediatrics, 2017; 139: e20164131. DOI: 10.1542/peds.2016-4131
7. Jones H, Kaltenbach K, Benjamin T, Wachman E, O'Grady K. Prenatal opioid exposure, neonatal abstinence syndrome/neonatal opioid withdrawal syndrome, and later child development research: shortcomings and solutions. J Add Med, 2019; 13: 90-2. DOI:10.1097/ADM.0000000 000000463

8. Jurand A. Teratogenic activity of methadone hydrochloride in mouse and chick embryos. J Embryol Exp Morphol, 1973; 30: 449-958.
9. Janas A, Folwarczna J. Opioid receptor agonists may favorably affect bone mechanical properties in rats with estrogen deficiency-induced osteoporosis. Naunyn-Schmiedeberg's Arch Pharmacol, 2017; 390: 175-85. DOI: 10.1007/s00210-0 16-1295-6
10. Stanhope T, Gill L, Rose C. Chronic opioid use during pregnancy: maternal and fetal implications. Clinics Perinatol, 2013; 40: 337-50.
11. Vijayendra K, Prasanna K, Harinath G. Evaluation of the efficacy of intrathecal fentanyl versus intrathecal nalbuphine as adjuvants to 0.75% ropivacaine for post-operative pain relief in cesarean section: a double-blind randomized comparative study. Int J Sci Study, 2017; 5: 5-10.
12. Amabile C, Bowman B. Overview of oral modified-release opioid products for the management of chronic pain. Ann Pharmacothe, 2006; 40: 1327-35. DOI:10.1345/aph.1G259
13. Wang H, Hsiong C, Ho S, Lin M, Shih T, Huang P, Hu O. 2014. Commonly used excipients modulate UDP-glucuronosyltransferase 2b7 activity to improve nalbuphine oral bioavailability in humans. Pharm Res, 2014; 31:1676-88. DOI:10.10 07/s11095-013-1272-4.
14. Kazemi M, Sahraei H, Azarnia M, Dehghani L, Bahadoran H, Tekieh E. The effect of morphine consumption on plasma corticosteron concentration and placenta development in pregnant rats. IJRM, 2011; 9: 71-6.
15. Karaboduk H, Uzunhisarcikli M, Kalender Y. Protective effects of sodium selenite and vitamin E on mercuric chloride-induced cardiotoxicity in male rats. Braz Arch Biol Technol, 2015; 58: 229-38. DOI.org/10.1590/S1516-8 91320140 0339
16. Zwolak I. The role of selenium in arsenic and cadmium toxicity: an updated review of scientific literature. Biol Trace Elem Res, 2019; 169: 121-8. DOI:10.1007/s 12011-019-01691-w
17. Shokraneh M, Sadeghi A, Mousavi S, Esmaeilkhanian S, Chamani M. Effects of in ovo injection of nano-selenium and nano-zinc oxide and high eggshell temperature during late incubation on antioxidant activity, thyroid and glucocorticoid hormones and some blood metabolites in broiler hatchlings. Acta Scientiarum. Animal Sciences, 2020; 42, e46029. DOI: 10.4025/actascianimsci.v42i1.46029.
18. Rayman M. Selenium and human health. Lancet, 2012; 379: 1256-68.
19. Todini A, Cosp A. Safety and efficacy of selenium compounds (E8) as feed additives for all animal species: sodium selenite. EFSA, 2016; 14: 4442.
20. Gueroui M, Kechrid Z. Evaluation of some biochemical parameters and brain oxidative stress in experimental rats exposed chronically to silver nitrate and the protective role of vitamin E and selenium. Toxicol Res, 2016; 32: 301-9.
21. Raza M, Deo C, Mandal A, Tyagi P, Mir N. Effect of dietary supplementation of selenium and vitamin E on growth performance and hematology of broiler chicken under heat stress. IJPS, 2018; 53: 171-6. DOI:10.5958/0974-8180.2018.00033.8
22. Turan B, Can B, Delilbasi E. Selenium combined with vitamin E and vitamin C restores structural alternations of bones in heparin-induced osteoporosis. J Clin Rheumatol, 2003; 22: 432-6.
23. Jakubovic A, MuGeer E, McGeer P. Effects of d1-methadone and morphine on developing chick embryo. Experientia, 1978; 34: 1617-8. DOI: 10.1007/bf02034 710
24. Paton N, Cantor A, Pescatore A, Ford M, Smith C. The effect of dietary selenium source and level on the uptake of selenium developing chick embryos. Poult Sci, 2002; 81: 1548-54. DOI: 10.1093/ps/81.10.1548
25. Cortés-Delgado N, Perez-Torres J, Hoyos J. Staining procedure of cartilage and skeleton in adult bats and rodents. Int J Morphol, 2009; 27: 1163-7. DOI.org/10.4067/S0717-95022009000400032.
26. Badawy G, Sakr S, Atallah M. Comparative study of the skeletogenesis of limb autopods in the developing chick Gallus domesticus and Toad Buforegularis. RJPBCS, 2012; 3: 966-88.
27. Nasiraei-Moghadam S, Kazeminezhad B, Dargahi L, Ahmadiani A. Maternal oral consumption of morphine increases bax/bcl-2 ratio and caspase 3 activity during early neural system development in rat embryos. J Mol Neurosci, 2010; 41: 156-64. DOI: 10.1007/s12031-009-9312-6
28. Salahshoor M, Khashiadeh M, Roshankhah S, Kakabaraei S and Jalili C. Protective effect of crocin on liver toxicity induced by morphine. RPS, 2016; 11: 120-9.
29. Källén B, Borg N, Reis M. The use of central nervous system active drugs during pregnancy.Pharmaceut, 2013; 6: 1221-86. DOI: 10.3390/ph6101221
30. Sadraiea S, Kakab G, Sahraeia H, Dashtnavard H, Bahadoran H, Mofid M, Nasab H and Jafar F. Effects of maternal oral administration of morphine sulfate on developing rat fetal cerebrum: A morphometrical evaluation. Brain Res, 2008; 1245: 36-40. DOI: 10.1016/j.brainres.2008.09.052.
31. Dehghan M, Jafarpour M, Mahmoudian A. The effect of morphine administration on structure and ultrastructure of uterus in pregnant mice. IJRM, 2010; 8: 111-8.
32. Ghafari S, Roshandel D, Golalipour M. Effect of intrauterine morphine sulfate exposure on cerebellar histomorphological changes in neonatal mice. Folia Neuropathol, 2011; 49: 328-34.
33. Niknam N, Azarnia M, Bahadoran H, Kazemi M, Tekieh E, Ranjbaran M, Sahraei H. Evaluating the effects of oral morphine on embryonic development of cerebellum in wistar rats. Basic Clin Neurosci, 2013; 4: 130-5.

34. Samarghandian S, Afshari R, Farkhondeh T. Effect of long-term treatment of morphine on enzymes, oxidative stress indices and antioxidant status in male rat liver. Int J Clin Exp Med, 2014; 7: 1449-53.
35. Shadid S, Barrett J. Opioid-induced osteoporosis: assessing causes and treatments. PPM, 2013; 13: 870-8.
36. Ezzatabadipour M, Majidi M, Malekpour-afshar R, Eftekharvaghefi S, Nematollahi-mahani S. The effects of morphine on tissue structure of the growth plate in male rats. Iran J Basic Med Sci, 2011; 14: 514-20.
37. Rozisky J, Dantas G, Adachi L, Alves V, Ferreira M, Sarkis J, Torres I. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int J Devl Neur, 2008; 26: 561-5. DOI:10.1016 /j.ijdevn eu.2008.05.005.
38. Beck T, Hapstack M, Beck K, Dix T. Therapeutic potential of kappa receptor agonists. Pharmaceut, 2019; 12: E95. DOI: 10.3390/ph12020095.
39. Li J, Gao R, Li S, Wang J, Tang Z, Xu S. Testicular toxicity induced by dietary cadmium in cocks and ameliorative effect by selenium. BioMetals, 2010; 23: 695-05. DOI: 10.1007/s10534-010-9334-0.
40. Surai P, Fisinin V. Feeding breeders to avoid oxidative stress in embryos. World´s Poultry Congress, 2012; 9: 1-12.
41. Liu H, Yu Q, Fang C, Chen S, Tang X, Ajuwon K, Fang R. Effect of selenium source and level on performance, egg quality, egg selenium content, and serum biochemical parameters in laying hens. Foods, 2020; 9: 68. DOI:10.3390/foods9010068
42. Zofkova I, Davis M, Blahos J. Trace elements have beneficial, as well as detrimental effects on bone homeostasis. Physiol Res, 2017; 66: 391-402. DOI:10.3354 9/physiolres.933454.
43. Zeng H, Cao J, Combs G. Selenium in bone health: roles in antioxidant protection and cell proliferation. Nutrients, 2013; 5: 97-110. DOI: 10.3390/nu5010097.