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THE EFFECTS OF ORAL ADMINISTRATION OF MORPHINE SULPHATE ON FOETUSES OF SPRAGUE-DAWLEY RATS *

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Abstract – The deleterious effects of morphine sulphate addiction on the central nervous system are well documented. Previous studies have shown that the passage of morphine from the placenta barrier can influence the normal development of embryos, such as those of humans, by specific mechanisms. So, for the first time, for the purpose of investigating the effects of morphine sulphate on pregnant animals, three groups (control, sham and experimental) of Sprague-Dawley female rats were chosen and 0.1, 0.2 and 0.3 mg/ml of morphine sulphate were administered orally in drinking water to each female rat (n=5-7) in four experimental groups in weeks 1, 2, 3 and 3 weeks of pregnancy. Caesarean sections were performed at the end of the gestation period; foetuses (n=27-63) and their placentas were examined externally; the number of foetuses and their resorption sites were also recorded. Results showed that 0.1, 0.2 and 0.3 mg/ml of morphine sulphate causes significant increase in the percentage of teratogenicity (except in week 3) ($p<0.05$). Although 0.1mg/ml of morphine did not have any effect on the diameter and weight of the placenta and the number of foetuses, 0.2 and 0.3 mg/ml of morphine caused a significant decrease ($p<0.05$) in the weight and diameter of placentas, the number of the embryos, their body weight and crown-rump length of fetuses. The foetal weight of all four groups decreased significantly ($p<0.05$). These results also showed that teratogenic effects of oral administration of morphine in rats mostly happens in week two (organogenesis) of embryonic development.

Keywords – morphine, teratogenicity, sprague-dawley rats, embryo

1. INTRODUCTION

By 1940, it was reported that the foetal malformations observed were genetic, but since 1942 studies have revealed that one of the most important causes of birth abnormalities is drugs passing through the placenta barrier.

During the 3rd century, morphine sulphate ($C_{17}H_{19}No_3$) was one of the drugs used for controlling diarrhea. It passes through the placenta gradually [1], but leaves the blood circulation very quickly and spreads into tissues such as lung, liver, kidney, spleen, brain and particularly adipose tissue [2, 3].

The injection of morphine sulphate and implantation of pellets into hen eggs, pregnant rats, mice, rabbits and sheep created qualitative, quantitative, structural and behavioural anomalies [1, 3-17].

Because of the stressful nature of procedures involving repeated injections or pellet implantation on specific days of pregnancy, morphine sulphate was orally administered via drinking water [1, 2, 9, 12, 17, 18] in Sprague-Dawley rats to determine: 1) effects of morphine on weeks 1, 2, 3 and 3 weeks of pregnancy, and 2) the most sensitive period of pregnancy.

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2. MATERIALS AND METHODS

Sprague-Dawley rats (200gr, 100-110 days old from the Razi Institute, Karaj, Tehran, Iran) were fed on laboratory food (pellets), housed at 20-25 ° C (room temperature) under a 12-hr light-dark cycle and 45-55% humidity. To reduce the risk of accidental abnormalities, second generation rats were used, with one male and one female rat in a cage from 4pm to 8am the following day. Lordosis behaviour was shown at the time of conception [12, 18-20].

0.1, 0.2 and 0.3 mg/ml of morphine sulphate (99.98%) in drinking water (25 ml) were administered orally [1, 18] with 0.5 gr/ml glucose (sucrose) added to the water to reduce its bitter taste [1, 12, 18, & 20].

Animals (n=5-7) were divided into three groups of control (cntl, using only drinking water), sham (using drinking water and sugar) and experimental rats (using drinking water, sugar and morphine) on weeks 1(w_1), 2(w_2), 3 (w_3) and 3 weeks (3w) of pregnancy.

Caesarean sections were performed at the end of the gestation period. Foetuses and placentas were examined externally and the number of foetuses and their resorption sites were also recorded. Foetuses were weighed to the nearest 0.01 gr on a torsion Sartorious balancer. Crown-rump (CR) length of foetuses and diameter of placentas were first measured with Koolis, and then fixed in Bouin's solution for soft tissue examinations and possible abnormalities.

Some of the normal and abnormal foetuses were processed, using alizarin red S and alcian blue 8GX staining to study the skeletal structures.

Statistical analysis

Parametric data (CR length, foetal body weight, placental weight and diameter) were analysed, using the one way ANOVA test. For determining the most sensitive period of pregnancy, the Tucky-Kramer test was used (q = 3.9). Nonparametric data (abnormalities) and a comparison of the percentages of normal and abnormal foetuses were analysed, using the chi squared (χ^2) test, where $p<0.05$ was considered as significant.

3. RESULTS

a) Effects of 0.1 mg/ml of morphine sulphate

I. Parametric (quantitative) data: The number of foetuses in the sham group (10.6 ± 0.8) exhibited no significant difference from the control (10.4 ± 0.7). In spite of a decline in the number of foetuses in all four experimental (treated) groups (9.2 ± 1.06, 10.6 ± 1.4, 9.8 ± 1.6 and 10.2 ± 1), the difference was not significant ($p<0.94$, $F=0.23$) compared to control rats.

Comparison of average foetal body weight in sham rats (5.65 ± 0.01gr) with that of control groups (5.66 ± 0.08gr) showed no significant difference, while experimental groups (except in w_1, 5.5 ± 0.1gr) showed significant differences ($p<0.04$, $F=1.63$) to control groups (Fig.1). Maximum decline in foetal body weight was observed in 3w, w_3, w_2 and w_1.

There was no significant difference between CR length in both control (4.3 ± 0.02 cm) and sham groups (4.26 ± 0.03 cm), but the average CR length in all treated groups (except w_3) showed a significant difference to control groups ($p<0.0001$, $F=29.04$, Fig. 1).

Average CR length in treated groups diminished in w_1, 3w, w_2 and w_3.

There was no difference between average placental diameter in sham (1.29 ± 0.02 cm) and control groups (1.26 ± 0.02 cm). The average diameter of the placenta of all treated groups (3w, w_1,

w_2 and w_3) showed no significant difference to the control, but the risk was maximum ($p<0.29$, $F=2.65$, Fig. 1).

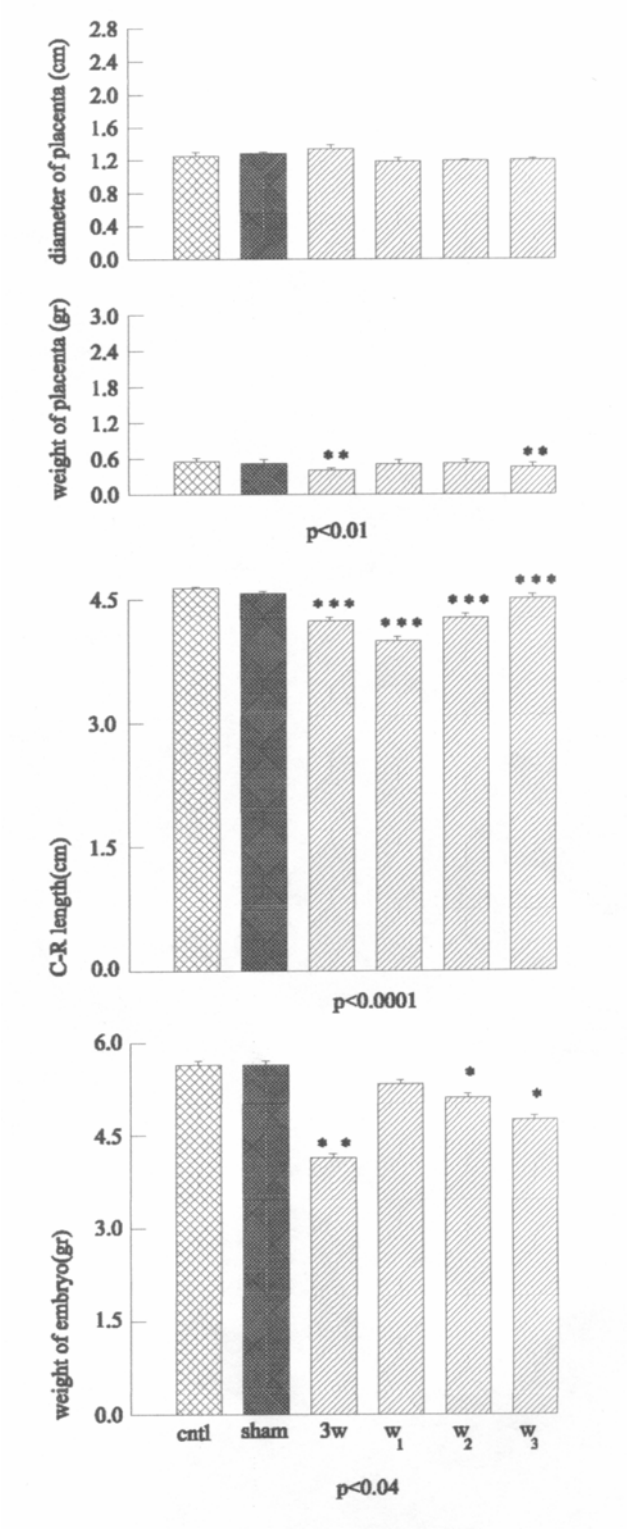


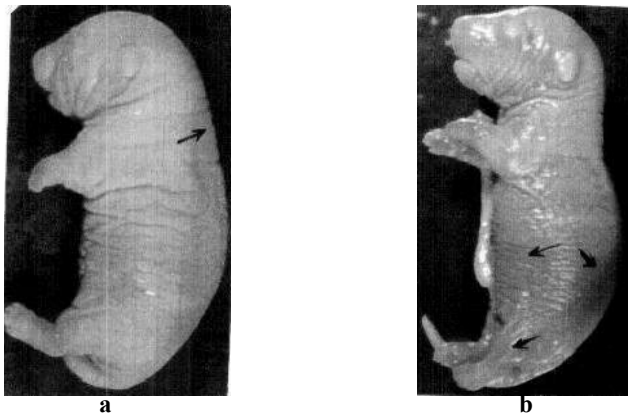
Fig 1. Effects of 0.1 mg/ml of morphine sulfate on parametric characteristics of 21-day old embryo of Sprague-Dawley rat

Average placental weight in the sham group (0.55 $\pm$0.401 gr) showed no difference to the control (0.56 $\pm$0.1 gr), while there was significant difference between two treated groups (3w and w_3), and the control ($p<0.1$, $F=12.4$, Fig. 1); the decline in the number of foetuses was pronounced in 3w, w_3, w_1 and w_2.

II. Nonparametric (qualitative) data: There was no significant difference in the percentage of abnormalities (atrophied embryo (a.e.) in 3w; cutaneous processes (c.p.) except in w_3; C-shaped embryo (c) except in w_1; abnormal curvature (a.c.); lack of normal curvature in the vertebral column (l.n.c.v.c.), except in w_3; deep depression in the vertebral column (d.d.v.c.), except in w_1; abnormal polarity in forelimb and hindlimb (a.p.f. and a.p.h.), except in w_3; subcutaneous haemorrhage (s.h.) and full blooded superficial blood vessels (f.b.s.b.v.), in w_1), between sham and control groups ($p<0.97$, $\chi^2=0.09$). Higher percentages of embryonic abnormalities (an .e.) occurred in w_2, 3w, w_1 and w_3, but it was only significant in w_2 and 3w ($p<0.0001$, $\chi^2=27.43$ and 22.7, Table 1, Fig.2).

Table 1. The effects of 0.1 mg/ml of morphine sulphate on weight of foetuses of rat, using Tucky-Kramer test. *$p<0.05$, $q>3.9$

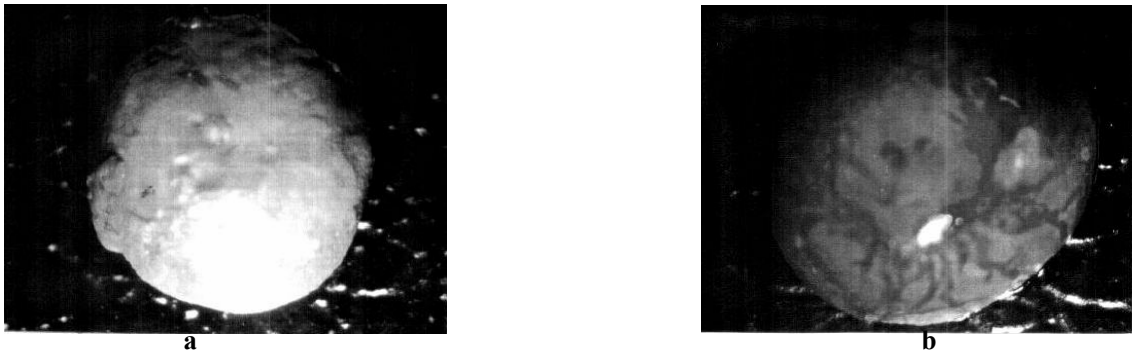
q	p	average (gr) $\pm$ SE	number	groups
-	-	5.66 $\pm$ 0.08	52	cntl
4.2*	<0.001	3.99 $\pm$ 0.02	46	3w
n.s 9.8	<0.1	5.5 $\pm$ 0.1	53	w_1
4.8*	<0.01	5.1 $\pm$ 0.1	49	w_2
7.9*	<0.01	4.76 $\pm$ 0.1	51	w_3



a **b**

Fig 2. 21- day old embryo of Sprague-Dawley rat: a) lack of normal curvature in vertebral column (arrow) and abnormality in the hindlimb polarity ; b) subcutaneous bleeding in the back of embryo(arrows)(10x)

No significant difference in placental abnormalities (light placenta haemorrhage (l.p.h.); small placenta (s.p.) and severe placenta haemorrhage (s.p.h.)) was observed between sham and control groups ($p<0.71$, $\chi^2=0.13$). There were s.p.h., l.p.h and fused placentas (f.p.) in w_1 and w_2, giant placenta (g.p., except in w_2) and s.p. in treated groups (Fig.3).



a b

Fig 3. 21- day old embryo of Sprague-Dawley rat: a) normal placenta
b) placenta with light hemorrhage(10x)

There was significant difference, with lower risk ($p<0.0001$, $\chi^2=15.19$ and 24.06), in 3w and w_1, high risk in w_2 ($p<0.01$, $\chi^2=6.7$) and maximum risk in w_3 ($p<0.04$, $\chi^2=4.2$).

In all experimental groups (except w_2), pregnant rats demonstrated vaginal haemorrhage (v.h.), abnormal uterus (a.u.) and early birth (e.b.). In spite of an increase in the percentage of abnormalities in all treated groups, it was only significant in group 3w ($p<0.03$, $\chi^2=3.75$), compared with the control.

III. Skeletal structures: Staining with alizarin red S and alcian blue 8GX showed no skeletal abnormalities in control, sham and treated (experimental) groups.

b) Effects of 0.2 mg/ml of morphine sulphate

I. Parametric (quantitative) data: There was a significant difference ($p<0.0001$, $F=12.3$) in the number of foetuses in 3w and w_1, compared with the control, but no variation between w_2, w_3 and the control .The decline in the number of foetuses was higher in 3w, w_1 and w_2. (Table 2), using the one way ANOVA test.

Table 2. The effects of 0.1 mg/ml of morphine sulphate on morphology of foetuses of rat (n=46-53), using Chi Square test. * $p<0.05$

groups abnormalities	cntl	sham	3w	w_1	w_2	w_3
a.e.	0.00001%	0.00001%	2.12%	0.00001%	0.00001%	0.00001%
c.p.	3.8%	1.9%	8.78%	1.9%	10.2%	0%
c	0.00001%	0.00001%	0.00001%	1.9%	0.00001%	0.00001%
a.c.	1.93%	1.9%	4.3%	1.9%	6.1%	0.00001%
l.n.c.v.c.	0.00001%	0.00001%	8.7%	3.8%	8.2%	0.00001%
a.p.f.	0.00001%	0.00001%	4.13%	1.9%	8.2%	0.00001%
a.p.h.	0.00001%	0.00001%	4.3%	1.9%	6.1%	2.16%
g.r.	0.00001%	0.00001%	0.00001%	0.00001%	0.00001%	0%
s.h.	1.9%	3.8%	19.6%	3.8%	20.4%	5.9%
f.b.s.b.v.	1.9%	0.00001%	0.00001%	1.9%	0.00001%	0%
d.d.v.c.	0.00001%	0.00001%	4.3%	0.00001%	2%	2%
an.e.	9.6%	7.5%	56.7%	18.8%	61.2	9.9%
P	-	<0.97	<0.0001	<0.28%	<0.0001	<0.97%
χ^2	-	0.0009n.s	22.7 **	1.57n.s	27.431 *	n.s 0.001

The difference between the average foetal body weight of all experimental and control groups was significant ($p<0.0001$, $F=29.7$, Fig. 4). The Tucky-Kramer test showed, 0.2 mg/ml of morphine sulphate most affected w_2, w_3, 3w and w_1.

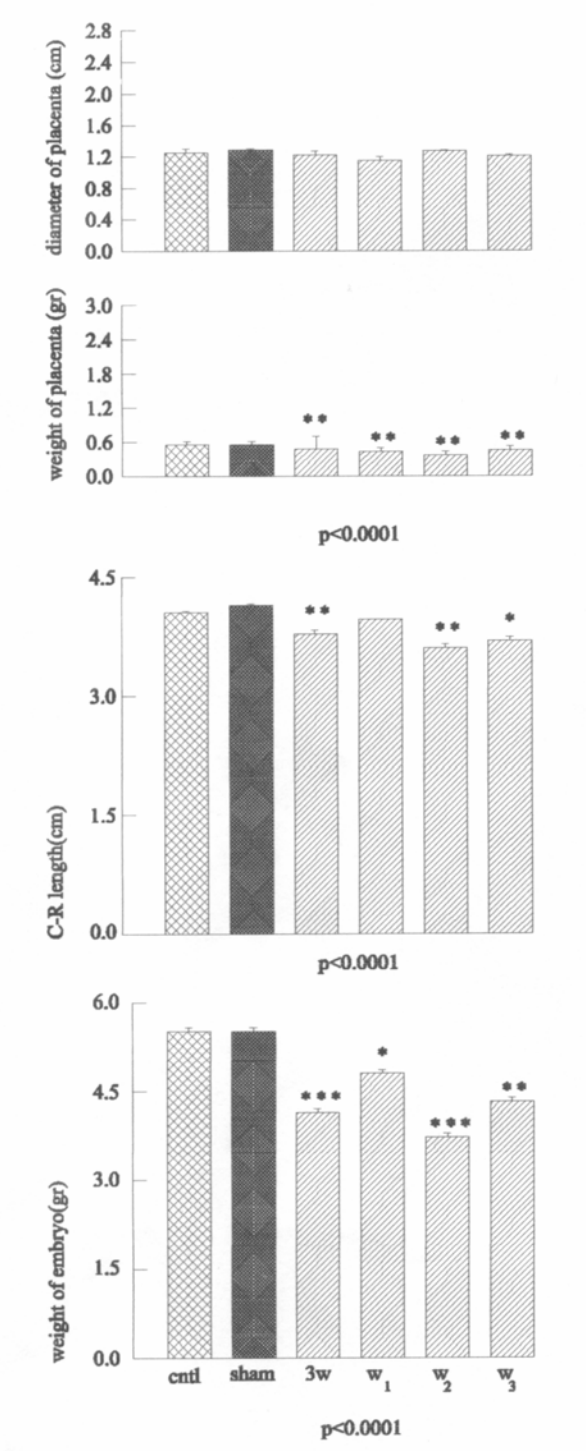


Fig 4. Effects of 0.2 mg/ml of morphine sulfate on parametric characteristics of 21-day old embryo of Sprague-Dawley rat

There was a significant difference between the CR length in three experimental groups (except in w_1, 4.01 cm $\pm$ 0.023) and the control (4.1 cm $\pm$ 0.02, $p<0.0001$, $F=32.26$, Fig. 4); 0.2 mg/ml of morphine most affected w_2, 3w, w_3 and w_1, using the Tucky-Kramer test.

Effects on the diameter of the placenta in the experimental groups was not significant ($p>0.5$, $F=0.78$), compared with the control (1.26 cm $\pm$ 0.01).

The weight of the placenta in all treated groups was diminished in w_2, w_3, w_1 and 3w, as measured, using the Tucky-Kramer test and the difference was significant ($p<0.0001$, $F=16.3$), compared with the control groups (Fig. 4).

II. Nonparametric (qualitative) data: Most abnormalities (a.e. in 3w and w_1; c.p., except w_2; a.c., in 3w and w_2; l.n.c.v.c., except 3w; a.p.f., in 3w and w_2; a.p.h., in w_1 and w_3; g.r., in 3w and w_1; s.h., in 3w; f.b.s.b.v., in 3w and w_3 and d.d.v.c. except w_3) were observed in w_3, w_2, w_1 and w_3. It was with minimal risk and significant in 3w ($p<0.0001$, $\chi^2=28.2$), with high risk and significant in w_2 ($p<0.001$, $\chi^2=9.9$) and with maximum risk but not significant in w_1 and w_3 ($p>0.05$).

The occurrence of placental abnormalities (s.p.h., except in w_2; l.p.h. and f.p., only in w_2; g.p., in 3w and w_3; and s.p., in w_1 and 3w) happened in 3w, w_1, w_3 and w_2, and was significant in 3w ($p<0.02$, $\chi^2=4.8$) and w_1 ($p<0.04$, $\chi^2=8.1$), compared with control groups.

There was a higher percentage of abnormalities, but not significant in w_3 ($p>0.7$, $\chi^2=0.13$), compared with the control .

The pregnant rats had a higher percentage of abnormalities (a.u., except in w_1; v.h., in w_2 and w_3; and e.b., in 3w and w_1) in experimental groups, with no significant difference compared with the control.

III. Skeletal structures: There was a delay in the ossification of lumbar and sacral vertebrae, but no apparent skeletal abnormalities occurred.

c) Effects of 0.3 mg/ml of morphine sulphate

I. Parametric (quantitative) data: The average number of foetuses was shown to diminish significantly in 3w and w_1 ($p<0.001$, $F=7.1$, Fig. 5), and happen in w_1, 3w, w_2 and w_3, using the Tucky-Kramer test.

The diminishing average foetus body weight of experimental groups was with minimum risk, and significant ($p<0.0001$, $F=108.3$) compared with the control. Maximum diminition of body weight was shown to occur in w_3, 3w, w_2 and w_1, respectively, using the Tucky-Kramer test.

According to the one way ANOVA test, the CR length had been reduced significantly ($p<0.0001$, $F=23.8$) in 3w, w_2, w_1 and w_3, with minimum risk in all treated groups, except in w_3 (Fig. 5).

Average placental weight was found to diminish significantly in all experimental groups, with minimal risk ($p<0.0001$, $F=24.03$, Fig. 5). The reduction occurred in w_3, w_2, w_1 and 3w, (Fig. 5), according to the Tucky-Kramer test.

There was no significant difference between the diameter of the placentas of experimental and control groups ($p>0.05$, $F=0.83$), using the one way ANOVA test (Fig. 5).

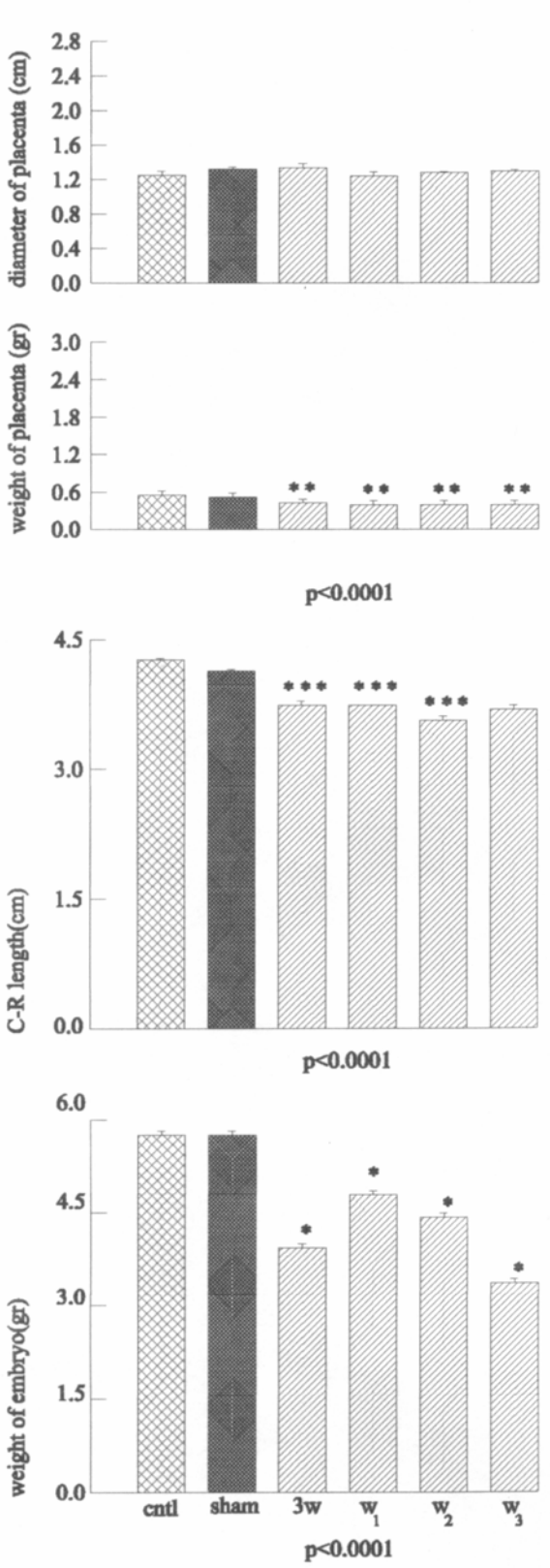


Fig 5. Effects of 0.3 mg/ml morphine sulfate on parametric characteristics of 21-day old embryo of Sprague-Dawley rat

II. Nonparametric (qualitative) data: The highest percentage of abnormalities (c.p., except in w_3; c, in 3w and w_2; ac.c. except in 3w; l.n.c.v.c., except in 3w; a.p.f., except in w_2; a.p.h., except in w_1; g.r., except in w_3; s.h., except in w_1 and w_3; f.b.s.b.v., except in 3w and w_1; and d.d.v.c., except in 3w and w_1) occurred in 3w, w_2, w_1 and w_3; The difference was not significant in w_3 ($p>0.9$, $\chi^2=0.004$), but was significant in 3w ($p<0.0002$, $\chi^2=13.9$), w_2($p<0.007$, $\chi^2=7$) and w_1($p<0.01$, $\chi^2=6.51$), compared with the control groups (Table 3).

Table 3. The effects of 0.2 mg/ml of morphine sulphate on length of crown-rump of foetuses of rat, using Tucky-Kramer test. *p<0.05, q>3.9

q	p	average (cm) ± SE	number	groups
-	-	4.11 ± 0.02	52	cntl
5.09 **	<0.01	3.85 ± 0.05	27	3w
2.73n.s	>0.05	4.01 ± 0.01	33	w_1
10.3 ***	<0.0001	3.67 ± 0.07	56	w_2
4.8 *	<0.02	3.95 ± 0.03	52	w_3

Abnormalities in placentas (s.p.h., except in w_1; l.s.h. and s.p.) were observed in 3w, w_1, w_2 and w_3, which were only significant in 3w ($p<0.02$, $\chi^2=5.1$) and w_1 ($p<0.03$, $\chi^2=4.5$), compared with the control.

Effects on pregnant rats created v.p. (3w), e.b. (except in w_2) and a.u. (except in w_2); The difference was not significant compared with control groups.

III. Skeletal structures: There were some delays in the process of ossification of the lumbar and sacral vertebrae, tibia, fibula and femur, in addition to the radius, ulna, humerus, and pterygoid (Fig.6).

a **b**

Fig 6. 21-day old embryo of Sprague-Dawley rat: a) normal ossification in forelimb ; b) delay in forelimb ossification (arrows), after staining with alizarin red S and alcian blue 8GX(10x)

4. DISCUSSION

Administered morphine sulphate decreases the number of foetuses because it induces the oestrus cycle, plasma and ovarian oestradiol, progesterone and weight [2, 5, 13, 19]. Morphine sulphate also blocks the release of FSH and LH [4, 19, 20], ovulation [5, 13, 19] and spermatogenesis [13]. By reducing cAMP expression, cellular response to hormones, cell division and cleavage are blocked, increasing the rate of embryonic death during implantation [5, 10, 21].

Decline in the number of foetuses only happened in w_1 and 3w; 0.1 mg/ml of morphine sulphate had very little influence on embryos, possibly because it would not affect cell division and more morphine may be needed [16, 17, 22].

Morphine sulphate created atrophied embryos in w_1 , because of the weakening of maternal immunity, causes embryonic development to cease during the early stages of implantation [23]. On the other hand, there is no placenta in the early stages and contact with the mother is through maternal

blood. The small size of litters creates a wider level of contacts with teratogens, causing embryonic resorption [20, 24].

Growth retardation [8, 16] is caused by a change in the structure of the placenta and reduction of FSH and LH [19]. It happened in w_1 and w_2, and there was a delay in the ossification of lumbar and sacral vertebrae.

The significant decrease in foetal body weight is possibly because of nutritional factors and/or level of growth hormones [16-17, 22, 25]. This is in agreement with the results of previous data presented [10, 17, 22 & 26].

Shortening of the CR length happened in all experimental groups, but there was no direct relation between the decline in foetal body weight and the shortening of CR length [26, 27]. Morphine decreases spaces between vertebrae and ribs [7, 28-30], and because they are cartilaginous and no ossification occurs at this stage, the vertebrae become closer and CR length becomes shorter.

Factors which decrease the weight and diameter of placentas are cell division arrest, reduction in cell size and angiogenesis. Larger embryos have larger placentas [1, 31]. Those who had giant placentas suffered severe superficial haemorrhage, which could be a sort of hyperplasia or hypertrophia compensating for blood circulation [14, 27, 32, 33]. The placenta is necessary for embryonic growth; defects could diminish the rate of growth and create embryonic abnormalities [12].

On day 11, rat embryos are C-shaped (c), and because it does not happen in group w_3, administration of morphine sulphate might have had delayed embryonic growth and maintained the shape until day 11.

Deviation of body axis and abnormal curvature in the vertebral column are being created by the effect of morphine on cell polarity and distribution of glycosaminoglycans [21]. Delay in ossification or abnormal growth of vertebrae could be the reason for axis deviation. Body axis is formed on day 8.5 and the rat is cylindrical. If some abnormalities appeared in group w_1 and w_2, it was not unexpected.

All types of haemorrhage were created because of a breakage of blood vessels, which was caused by the effect of morphine on angiogenesis [2, 14].

Administration of morphine in w_2 created the highest percentage of abnormalities. Morphine sulphate can be stored in skeletal muscles, liver, kidney and the lungs [2, 3, 10, and 14] and used in week two to cause anomalies. Week 3 is a period of organ growth; although it is not significant, there is a higher decrease in foetal body weight in w_3.

The influence of morphine on the extra cellular matrix could interfere with the normal development of the embryo and create anomalies [21]. While abnormalities happen in specific periods of development, it is different in the case of the placenta; a longer period of exposure to morphine creates more abnormalities in placentas.

Pregnant rats also had some abnormalities. Ciociola [1, 30] reported that morphine sulphate affects uterus smooth muscle and its contraction [7], and premature birth is caused by the effect of morphine on the level of FSH and LH.

At the skeletal level, there were delays in ossification. Morphine sulphate blocks gene expression in osteoblasts [14].

Results clearly confirm the effects of morphine sulphate on Sprague-Dawley rat embryos, although the mechanisms of its influence must be investigated intensively.

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