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Age- and sex-related changes in the severity of physical and psychological dependence in morphine-dependent rats

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ABSTRACT

Gender- and age-dependent effects on the severity of morphine dependence are still controversial. The aim of this study was to investigate the effects of age and sex on the severity of physical and psychological dependence in morphine-dependent rats. The adult/aged male and female Wistar rats were chronically treated with bi-daily doses (10 mg/kg, at 12 h intervals) of morphine for 14 days. Then, rats were tested for the severity of physical dependence on morphine (spontaneous withdrawal signs), anxiety-like (the elevated plus maze), depressive-like (sucrose preference test) and grooming behaviors after spontaneous morphine withdrawal. We found that the morphine withdrawal signs decreased after 3 and 7 days of withdrawal in female and male rats respectively, while there was no significant difference in overall dependence severity between the two sexes or ages. Also, we found that the withdrawal of morphine led to increased anxiety, depression and obsessive-compulsive behavior in the D (dependent)/Adult male and female rats. Also, the D/aged female and male rats exhibited a reduction in depressive-like behavior than the D/Adult rats. Moreover, the D/female rats exhibited a decreased obsessive-compulsive behavior in both age groups than male rats. We conclude that age has no effect on the duration of withdrawal from morphine and overall dependence severity. While, the duration of withdrawal from morphine was lower in female than male rats. Our results showed a sex difference on the duration of morphine withdrawal and an age difference in the expression of psychological dependence on morphine. Thus, therapeutic strategies may be different for opiate-dependent individuals in physical and psychological dimensions.

1. Introduction

The annual rate of opioid users was 0.6%– 0.8% of the global population, mainly in people aged 15–64 years. This number includes 13.5 million to opiates (Pacurucu-Castillo et al., 2019). Drug abuse is a growing problem among men and women, but with different treatment programs that the amount and quality of the information are lacking (Langan and Pelissier, 2001; Pacurucu-Castillo et al., 2019). Sex differences have been studied extensively in drug addiction. However, there remains considerable controversy in this regard, including sex- and age-related effects on the severity of physical and psychological dependence in animal and human studies of drug addiction. For example, it has been reported that male rats showed more analgesic activity (Cicero et al., 1996), severe withdrawal symptoms (Cicero et al., 2002), tolerance to morphine (Barrett et al., 2001), a decrease in depression (Hodgson et al., 2010) and anxiety-like behavior (Pedrón et al., 2016) during morphine withdrawal. On the other hand, female rats revealed more morphine-induced conditioned place preference (CPP) (Randall et al., 1998), heroin self-administration (Cicero et al., 2003) and robust locomotor sensitization to psychostimulants (McCormick et al., 2005). In human studies, men used more heroin (Palamar et al., 2016); while, women have been reported to have more craving (Back et al., 2011), sadness and sensitivity to cue-induced relapse following heroin use (Yu et al., 2007), and also higher rates of depression and anxiety (Green et al., 2009). Drug addiction in older adults is often neglected (Bhatia et al., 2015; Carew and Comiskey, 2017; Maree et al., 2016) that results in serious health complications (Chang, 2018). Considering the increasing prevalence and proportion of substance use in the elderly (Carew and Comiskey, 2017), and also significant and growing use of opioids by women (Craft, 2008), it is noteworthy that sex likely interacts with age in differential sensitivities mentioned above. For example, chronic social stress during adulthood did not increase the amphetamine-induced CPP in female rats (Mathews et al., 2008). Also, methamphetamine self-administration changed 5-

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HT2CR localization in adult males (Hankosky et al., 2018). Furthermore, acute morphine withdrawal signs were more in the adults, but no sex differences were observed (Radke et al., 2015). In contrast, another study suggests that sex difference in drug abuse is more evident in adults (Buccelli et al., 2016). The nigrostriatal and the mesolimbic systems are vulnerable to aging (Cruz-Muros et al., 2007), and a selective impairment of nigrostriatal and mesolimbic dopamine occurs in aging rats (Friedemann and Gerhardt, 1992; Miguez et al., 1999). The dynamic nature of brain function, however, suggests that substance abuse during older adults may increase the risk of addiction (Dowling et al., 2008). So, it is notable that there is a lack of knowledge about sex- and age-related differences in opioid dependence and withdrawal, especially in adult and aged rats, which in turn, influences the treatment strategies. Therefore, the aim of this study was to investigate the effects of sex and age on the severity of physical dependence, anxiety, depression and obsessive-compulsive-like behaviors following the development of morphine dependence on adult and aged male and female rats.

2. Material and method

2.1. Animals and induction of morphine dependence

The adults (4–5 months old, $n = 24$) and aged (18–19 months old, $n = 28$) male and female Wistar rats, born and bred in our laboratory, were housed in cages in groups of 3 or 4 with the same sex and age on a 12-h light/dark cycle at 22–24 °C and food and water were available ad libitum. All experimental trials were conducted in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Morphine sulfate (Temad, Iran) were injected subcutaneously at a dose of 10 mg/kg, twice per day at 12 h intervals as described previously (Mokhtari-Zaer et al., 2014) for 14 days. Control rats were treated similarly with injections of saline. Rats were divided randomly into four groups ($n = 6$–7 per group): Saline-Adult (Sal/Adult), Saline-Aged (Sal/Aged), Dependent-Adult (D/Adult) and Dependent-Aged (D/Aged), including both male and female sexes.

2.2. Spontaneous withdrawal signs

Withdrawal signs were recorded and scored daily during a period of 30 min for 10 consecutive days from day 15 (24 h after the last injection of morphine) to day 24 of morphine withdrawal (see Fig. 1; Timeline of the experiment) according to a modified version of the Gellert–Holtzman scale, as described previously (Mokhtari-Zaer et al., 2014). Briefly, each rat was placed in a transparent cylinder (35 cm diameter, 50 cm height), and its behavior was monitored by a rater blinded to the rats' treatment. On the Gellert and Holtzman scale, graded signs including jumps, wet dog shakes, abdominal contractions were counted as the number of events occurring during the test time and assigned a weighting factor 1–4 based on the frequency of appearance, Body weights of each rat was monitored daily over 10 days, and the percentage of body weights changes was calculated from 1 day before to 1 day after. Checked signs including diarrhea, ptosis, erection or genital grooming, teeth chattering, writhing, and irritability received a factor range of 2–3 for their presence. Checked signs were counted as positive if the sign occurred at any time during the observation period. After completion of the observation session, the overall withdrawal severity was calculated by summing the proper weighting factor of somatic signs.

2.3. Anxiety measurement in the EPM (elevated plus maze test)

For the assessment of anxiety, the rats were individually placed in the center of the EPM 11 days after morphine withdrawal (day 25) (see Fig. 1; Timeline of the experiment), as described previously (Mokhtari-Zaer et al., 2014). Time spent in, and entries into the open and closed arms were measured during each 5-min test by a tracking system (EthoVision, Noldus, The Netherlands). The apparatus was cleaned after each trial with water.

2.4. Grooming behavior for the assessment of obsessive–compulsive disorder (OCD)

Rats were individually placed in a Plexiglas box (41 cm length × 33 cm height × 41 cm width), and grooming behavior as OCD-like behavior (Georgiadou et al., 2012) was evaluated on day 26 (see Fig. 1; Timeline of the experiment). Components of grooming behavior included vibration, face and head washing, body grooming, scratching, paw licking, head shaking and genital grooming (Georgiadou et al., 2012; Graf et al., 2003). Grooming behavior was scored every 15 s for 30 min. Thus, the maximum of the available score during the observation period was 120, and considered as an index of obsessive-compulsive disorder, as described previously (Georgiadou et al., 2012; Graf et al., 2003; Hammami-Abrand Abadi and Miladi-Gorji, 2016).

2.5. Sucrose preference test (SPT) to assess depressive-like behavior

All rats were maintained in individual cages for 24 h before testing. A modified model of the SPT as previously described (Fadaei et al., 2015) was carried out as follows from day 27 to day 28 (see Fig. 1; Timeline of experiment). Rats were allowed continuous access to two bottles in each cage for 48 h, one with 200 ml of 32% sucrose (*w/v*) and the other also with 200 ml of tap water. The positions of the bottles were changed every 12 h to avoid learning. Fluid and sucrose intake were measured every day. At the end of 48 h, the bottles were removed and sucrose preference was calculated as: 100% × sucrose solution consumption (ml)/total fluid consumption (ml) (Sáenz et al., 2006).

2.6. Statistical analysis

The data were expressed as the mean ± SEM. Data exhibited a normal distribution and were analyzed using three-way analyses of variance (ANOVA) with the fixed factors dependence × sex × age, with repeated measures as required. Post-hoc analyses included Tukey's test. Statistical differences were considered significant at $P < 0.05$.

3. Results

3.1. The spontaneous withdrawal signs

The results of the signs of spontaneous morphine withdrawal are

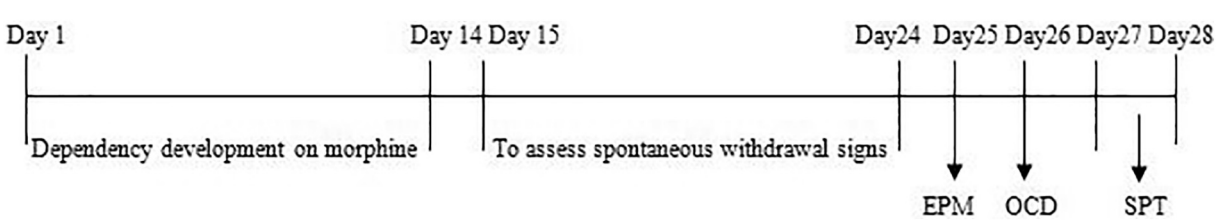

Fig. 1. Time lines of the experiments (see Materials and methods for details).

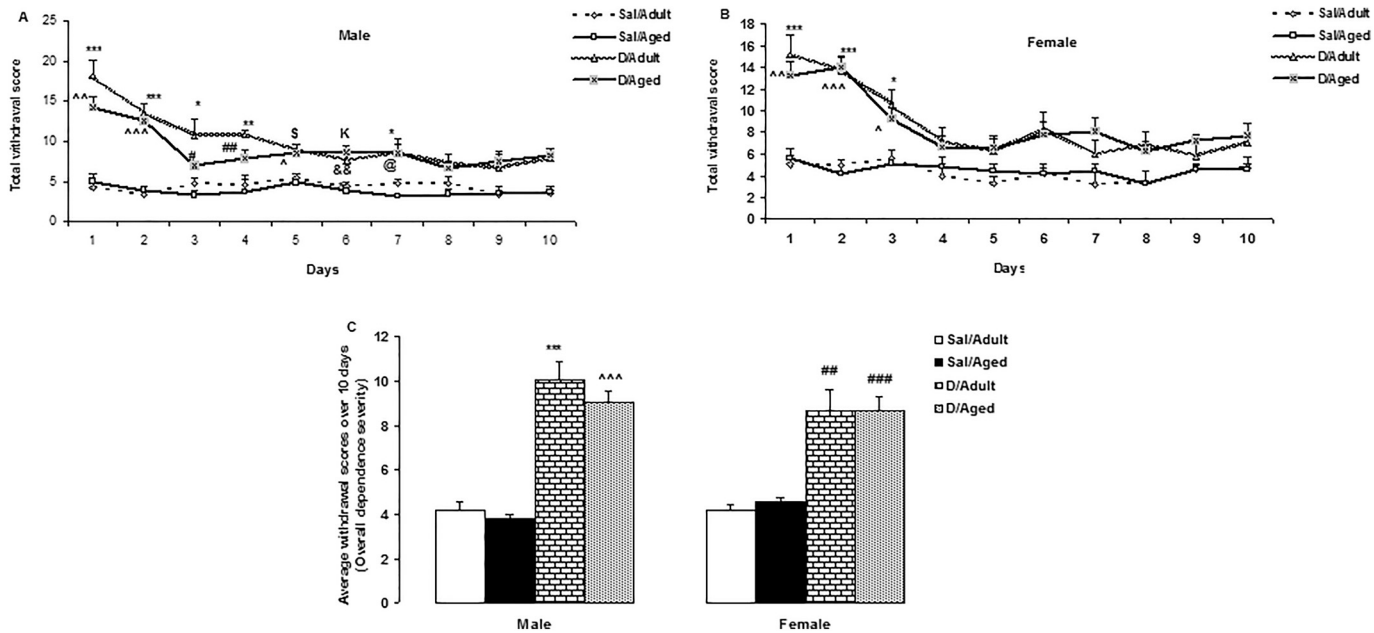

Fig. 2. The spontaneous withdrawal signs in morphine dependent adult and aged male and female rats. (A) Total withdrawal score in male. (B) Total withdrawal score in female. (C) Average withdrawal scores over 10 days. Morphine withdrawal signs in morphine dependent adult and aged rats decreased during seven (in male) and three days (in female). Also, there was no significant difference on the severity of morphine dependence between two the sexes or ages. In (A):$^{***}P = 0.0001$, $^{***}P = 0.0001$, $^{*}P = 0.011$, $^{**}P = 0.0001$, $^{\$}P = 0.05$, $^{K}P = 0.05$, $^{*}P = 0.039$ vs. Sal/Adult male group; and $^{\wedge\wedge}P = 0.001$, $^{\wedge\wedge\wedge}P = 0.0001$, $^{\#}P = 0.033$, $^{\#\#}P = 0.009$, $^{\wedge}P = 0.038$, $^{\&\&}P = 0.003$, $^{@}P = 0.024$ vs. Sal/aged male group. In (B): $^{***}P = 0.0001$, $^{***}P = 0.0001$, $^{*}P = 0.04$ vs. Sal/Adult female group; and $^{\wedge\wedge}P = 0.002$, $^{\wedge\wedge\wedge}P = 0.0001$, $^{\wedge}P = 0.05$ vs. Sal/aged female group. In (C): $^{***}P = 0.0001$ vs. Sal/Adult male group, $^{\wedge\wedge\wedge}P = 0.0001$ vs. Sal/aged male group, $^{\#\#}P = 0.0001$ vs. Sal/Adult female group, $^{\#\#\#}P = 0.0.001$ vs. Sal/aged female group.

shown in Fig. 2. Analysis of withdrawal signs using thee-way ANOVA with repeated measure revealed significant effects of day ($F_{9,378} = 23.3$, $P = 0.0001$), dependence ($F_{1,42} = 122.5$, $P = 0.0001$), and significant interactions between day × sex ($F_{9,378} = 3.1$, $P = 0.045$), day × dependence ($F_{9,378} = 15.91$, $P = 0.0001$) and day × age × dependence ($F_{9,378} = 3.84$, $P = 0.05$). As shown, the morphine withdrawal signs in morphine dependent adult and aged male rats (Fig. 2A.) decreased over a 7-day withdrawal period. The between-group comparisons indicated significant difference between the D/Adult and Sal/Adult male rats on days 1 ($P = 0.0001$), 2 ($P = 0.0001$), 3 ($P = 0.011$), 4 ($P = 0.0001$), 5 ($P = 0.05$), 6 ($P = 0.05$) and 7 ($P = 0.039$). Also, there was significant difference between the D/aged and Sal/aged male rats on days 1 ($P = 0.001$), 2 ($P = 0.0001$), 3 ($P = 0.033$), 4 ($P = 0.009$), 5 ($P = 0.038$), 6 ($P = 0.003$) and 7 ($P = 0.024$). However, the morphine withdrawal signs in morphine dependent young and aged female rats (Fig. 2B) decreased over a 3-day withdrawal period. The between-group comparisons indicated significant difference between the D/Adult and Sal/Adult female rats on days 1–3 ($P = 0.0001$, $P = 0.0001$ and $P = 0.04$, respectively) and the D/aged and Sal/aged female rats on days 1–3 ($P = 0.002$, $P = 0.0001$ and $P = 0.05$, respectively).

Three-way ANOVA analysis of the average withdrawal scores over 10 days (overall dependence severity) revealed no significant effect of age ($F_{1,42} = 0.38$, $P = 0.53$), and sex ($F_{1,42} = 0.57$, $P = 0.45$); but significant effect of treatment ($F_{1,42} = 120.16$, $P = 0.0001$); and also no significant interaction between age × sex × treatment ($F_{1,42} = 0.032$, $P = 0.85$). So, there was no significant difference on the severity of morphine dependence between two the sexes or ages. Pair-wise Bonferroni comparisons (adjusted alpha = 0.025) revealed that overall dependence severity was significantly more in the D/Adult ($P = 0.0001$, in both sexes) and D/aged ($P = 0.0001$ in male and $P = 0.001$ in female) than their control groups (Fig. 2C).

3.2. The anxiety-like behavior

The results of the elevated plus maze (EPM) are illustrated in Fig. 3A. Three-way ANOVA revealed no significant effects of age ($F_{1,41} = 0.73$, $P = 0.39$), and sex ($F_{1,41} = 1.67$, $P = 0.2$) but significant effects of dependence ($F_{1,41} = 22.99$, $P = 0.0001$), and sex × dependence interaction ($F_{1,41} = 4.9$, $P = 0.033$) for open arm time. Pair-wise Bonferroni comparisons (adjusted alpha = 0.025) revealed that the time spent on the open arms was significantly less in the D/Adult male ($P = 0.043$), and also the D/Adult ($P = 0.008$) and D/aged ($P = 0.022$) female rats than their control groups. There was no significant statistical difference in total arm entries.

3.3. Depression and obsessive-like behaviors

Three-way ANOVA analysis of the percentage of sucrose preference revealed significant effects of age ($F_{1,42} = 25.66$, $P = 0.0001$), dependence ($F_{1,42} = 9.22$, $P = 0.004$), and age × dependence ($F_{1,42} = 10.99$, $P = 0.002$) interaction. Between-group comparison also revealed that the D/Adult male ($P = 0.034$) and female ($P = 0.011$) rats had less sucrose preference than their control groups. The percentage of sucrose preference in aged morphine-dependent rats was significantly higher than adult morphine-dependent rats in both male ($P = 0.003$) and female ($P = 0.001$); while, they were not significantly different from their control groups (Sal/aged rats) (Fig. 4A).

Results of the self-grooming behavior are shown in Fig. 4B. Three-way ANOVA revealed significant effects of sex ($F_{1,42} = 43$, $P = 0.0001$), dependence ($F_{1,42} = 9.22$, $P = 0.004$), age × dependence ($F_{1,42} = 7.73$, $P = 0.008$) and sex × dependence ($F_{1,42} = 4.98$, $P = 0.031$) interaction. The self-grooming score in the D/Adult male ($P = 0.003$) and female ($P = 0.039$) rats were significantly higher than their control groups (Sal/Adult rats). Also, the self-grooming score was lower in the D/Adult ($P = 0.0001$) and D/Aged ($P = 0.001$) female rats than their control groups in male rats.

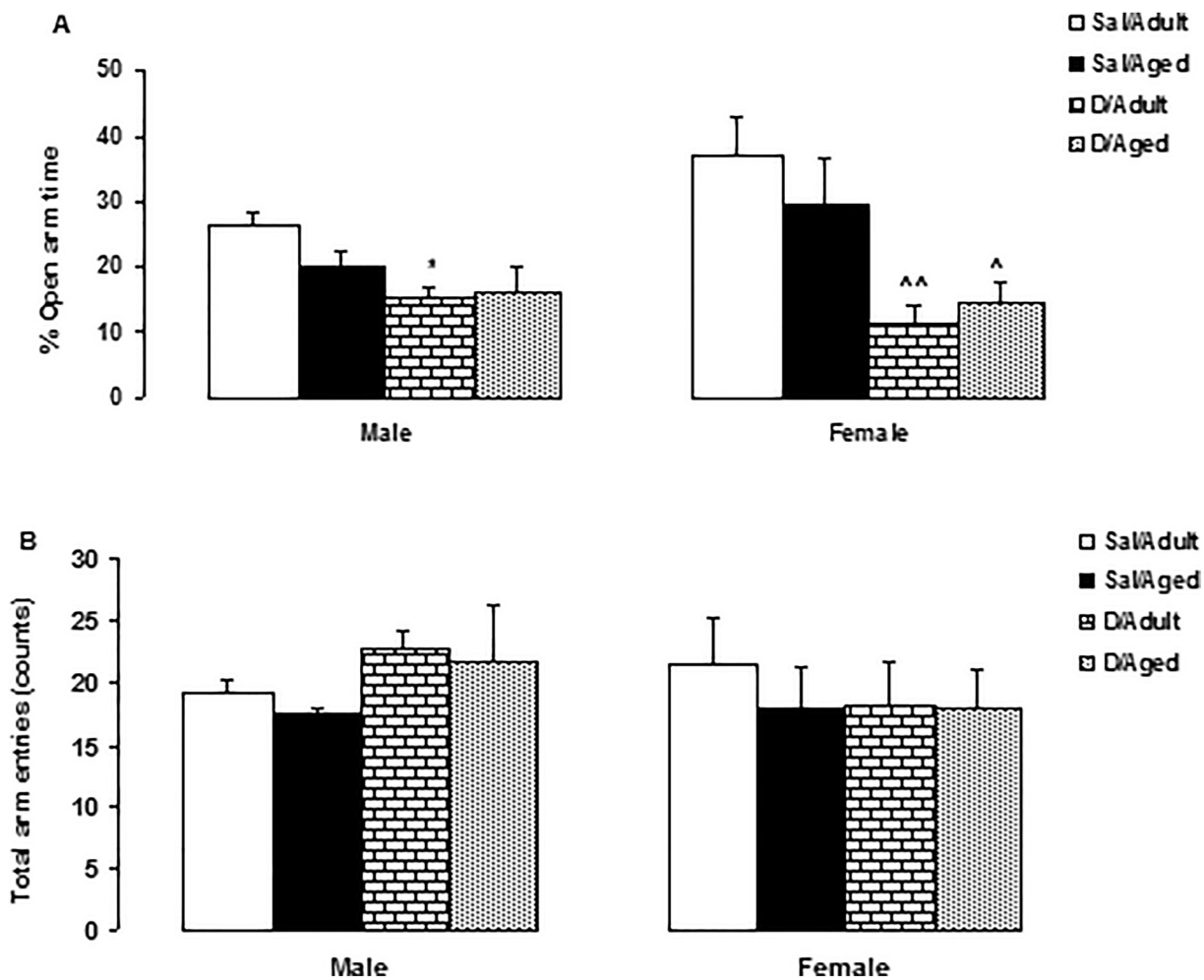

Fig. 3. The anxiety-like behavior after morphine withdrawal in morphine dependent adult and aged male and female rats. (A)The percentage of time spent in the open arm of the EPM in male and female. (B) The number of total arm entries in male and female. The D/Adult male and D/Adult and D/aged female rats had significantly more anxiety than their control rats. $^{*}P = 0.043$ vs. Sal/Adult, $^{\tilde{}}P = 0.008$ vs. Sal/Adult and $^{\hat{}}P = 0.022$ vs. Sal/aged.

4. Discussion

4.1. The severity of physical dependence

The results of our study indicated that there was no significant difference in the severity of physical dependence on morphine (the average of spontaneous withdrawal score over 10 days according to the Gellert-Holtzman rating score) between adult and aged rats of both sexes. The reason for this finding is not clear, but it seems that there is a similar biological basis for the development of plastic changes in the dopaminergic circuitry of the adult and aged rats of both sexes following chronic administration of morphine. Furthermore, consistent with this result, no sex differences have been observed in the severity of heroin and opioid withdrawal symptoms between men and women (Riley et al., 2018). It was previously reported that there were no differences between male and female rats in the time course or magnitude of acute morphine withdrawal-induced startle (Radke et al., 2015). This lack of difference may be because of the similarity of morphine serum and brain concentration in both sexes rats (Cicero et al., 1997).

Also, we found that the withdrawal signs were considerably more prolonged in males (for up to 7 days) than in females (for up to 3 days) after withdrawal. In contrast to our findings, it has been shown that there are sex differences only in the expression of the spontaneous withdrawal signs, and male rats had more severe withdrawal signs than females for up to 4 days after withdrawal (Cicero et al., 2002). However, we have previously shown that the signs of spontaneous morphine withdrawal lasted up to 7 days in adult male rats (Hammami-Abrand Abadi and Miladi-Gorji, 2016; Mokhtari-Zaer et al., 2014). Such discrepancy may arise from doses of morphine (with an escalation to a final dose of 40 mg/kg twice daily), age and/or race of rats (which were Sprague–Dawley rats with 60–80 days of age) (Cicero et al., 2002). However, the male and female Wistar rats used in this study were adult (4–5 months old) and aged (18–19 months old). Also, in another study, males were more sensitive to naloxone-precipitated morphine withdrawal than females (Pedrón et al., 2016) and experienced longer spontaneous withdrawal symptoms (Riley et al., 2018). This difference in the length of withdrawal may be due to processes which underlie synaptic alterations with age which may be different in males and females (Messing et al., 1981) and also a greater sensitivity to morphine in male (Pedrón et al., 2013).

Previous studies have shown that female rats may be more vulnerable to the rewarding properties of opioids (Cicero et al., 2003; Fattore et al., 2009; Randall et al., 1998), and ovarian hormones probably play an important role in such differences especially in adult rats (Fattore et al., 2009). However, our results suggest that morphine withdrawal takes a shorter time in female rats. No study with the same nature has been conducted thus far. This finding is consistent with previous results showing that female rats become addicted faster to drugs and respond better to treatment than males, which may be due to differences in the development of reward circuitry in males and females (Carroll et al., 2004), and intrinsic sex-related differences in the sensitivity of the brain to morphine (Cicero et al., 2002). So, it has been suggested that

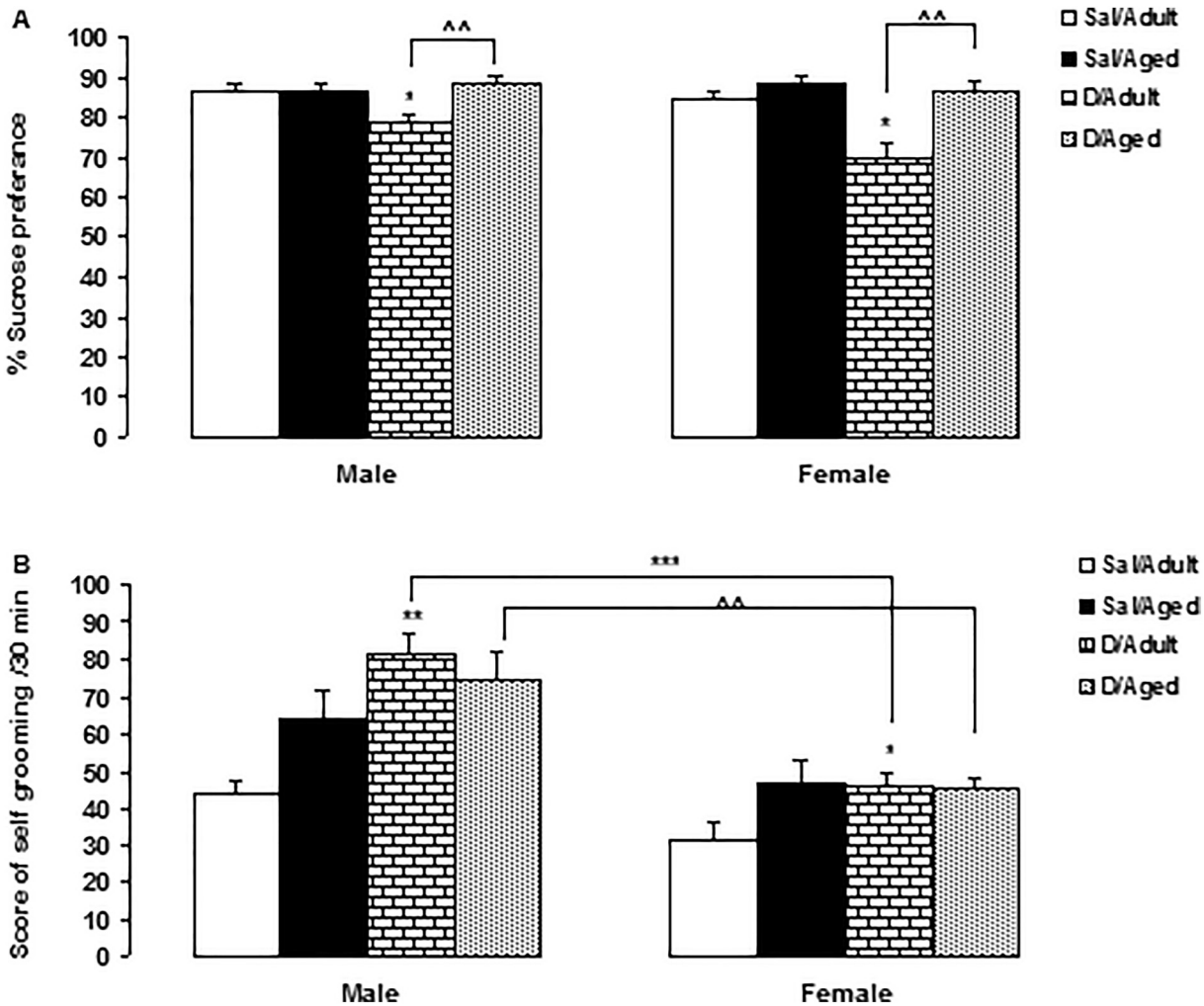

Fig. 4. The depression and obsessive-like behaviors after morphine withdrawal in morphine dependent adult and aged male and female rats. (A) The percentage of sucrose preference. (B) Self-grooming signs. The percentage of sucrose preference in D/Adult groups was significantly less than Sal/Adult and D/aged groups in both male and female. Also, the self-grooming score in the D/Adult male and female rats were significantly higher than Sal/Adult rats. In (A):$^{*}P = 0.034$, $^{\hat{}}P = 0.011$ vs. Sal/Adult group; $^{**}P = 0.003$, $^{\hat{}\hat{}}P = 0.001$ D/Adult group. In (B): $^{**}P = 0.003$, $^{*}P = 0.039$ vs. Sal/Adult group; $^{***}P = 0.0001$ vs. D/Adult male group; $^{\hat{}}P = 0.001$ D/aged male group.

addicted women need different treatments than men (Evans and Foltin, 2010). Also, in the present study, the lack of significant differences in the severity of physical dependence between adult and aged female rats may be due to the destruction of the estrous cycle of females following chronic administration of morphine (Craft et al., 1999). The differences observed between sexes in our study cannot be related to baseline measurements in each sex or age, since there were no significant differences between the male and female/adult and old saline control groups. Thus, the differences observed apparently reflect intrinsic sex- and/or age-related differences in the development of the morphine withdrawal.

4.2. The anxiety, depression, and obsessive-like behaviors

We found that the withdrawal of morphine exhibited the anxiety/depression and obsessive-like behaviors in the D/Adult male and female rats. Also, the D/aged female rats exhibited the anxiety-like behavior. No study with the same nature has been conducted thus far. Our finding is in agreement with our previous studies showing that morphine withdrawal is associated with an increase in the anxiety (Fadaei et al., 2015; Ghodrati-Jaldbakhan et al., 2017) in adult male and female, depression (Fadaei et al., 2015) and grooming behavior (Hammami-Abrand Abadi and Miladi-Gorji, 2016) in adult male rats. The dysphoric state associated with morphine withdrawal in the present study may be due to activation of brain stress systems (Koob, 2009), increase of the corticotropin-releasing factor (CRF) in the amygdale (Heinrichs et al., 1995), decrease of the brain concentration of norepinephrine (Paulson et al., 1991), serotonin (Mitsukawa et al., 2010) and dopamine (Diana et al., 1995). It has been proved that the anxiety/depression and obsessive-like behaviors produced by morphine withdrawal are involved in higher susceptibility to relapse following protracted abstinence from opiate (Self and Nestler, 1998).

The D/aged rats were more resistant to depression and obsessive-like behaviors after morphine withdrawal than adult rats. In the present study, we found a significant age difference and no sex difference in the expression of both OCD and depression, indicating high comorbidity of OCD with depression and common neurobiological mechanisms (Cardoner et al., 2007). This may be through adaptation and habituation to repeated stress following morphine withdrawal in the D/aged male and female rats. It is consistent with a study (Shoji and Mizoguchi, 2010) which reported that stress-induced c-Fos expression decreased in many brain areas of aged rats that are involved in the stress response and also there was no significant difference in serum levels of corticosterone in adult and aged rats. Our findings suggest that the behavioral disorders observed at this age of rats may be independent of sex hormones.

We also found no significant difference in the anxiety-like behavior between two sexes or ages of rats. Moreover, the depression-like behavior did not differ between two sexes. These findings might be helpful in understanding the lack of significance in the severity of physical dependence on morphine between adult and aged rats of both sexes. It has been shown that older patients achieve better treatment outcomes than their younger counterparts (Carew and Comiskey, 2017), which may be due to decreased obsessive-compulsive behavior in the elderly, which was observed in this study.

In general, many factors may be involved in the behavioral disorders observed, which is a limitation of our study. Although we tried to reduce the effects of extraneous variables.

5. Conclusion

Our results showed that there was no significant difference in the severity of physical dependence on morphine between adult and aged rats of both sexes. Also, morphine withdrawal takes a shorter time in female rats. Our results showed a sex difference on the duration of morphine withdrawal and an age difference in the expression of psychological dependence on morphine. As, the D/aged rats of both sexes did not exhibit the depression and obsessive-like behaviors after morphine withdrawal. Our findings suggest that the therapeutic strategy may be different in addicted individuals in both two sexes and age, regarding the physical and psychological aspects of addiction.

Declaration of competing interest

The authors report no conflicts of interest.

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