Oleic Acid Produces Motor Incoordination and Hypoactivity in Infant Wistar Rats Through GABA_A Receptors

Gabriel Guillén-Ruiz¹, Blandina Bernal-Morales¹, Carlos M. Contreras¹,²,*, Jonathan Cueto-Escobedo¹, Juan Francisco Rodríguez-Landa¹

¹Neuropharmacology Section, Institute of Neuroethology, University of Veracruz, Xalapa, Veracruz, México
²Peripheral Unit, Biomedical Research Institute, National Autonomous University of México, Xalapa, Veracruz, México

Email address: gguillen@uv.mx (G. Guillén-Ruiz), bbernal@uv.mx (B. Bernal-Morales), ccontreras@uv.mx (C. M. Contreras), jcueto@uv.mx (J. Cueto-Escobedo), juarodriguez@uv.mx (J. F. Rodríguez-Landa)
*Corresponding author

To cite this article: Gabriel Guillén-Ruiz, Blandina Bernal-Morales, Carlos M. Contreras, Jonathan Cueto-Escobedo, Juan Francisco Rodríguez-Landa. Oleic Acid Produces Motor Incoordination and Hypoactivity in Infant Wistar Rats Through GABA_A Receptors. American Journal of Psychiatry and Neuroscience. Vol. 4, No. 2, 2016 pp. 18-25. doi: 10.11648/j.ajpn.20160402.11

Received: March 8, 2016; Accepted: March 18, 2016; Published: March 31, 2016

Abstract: A mixture of eight fatty acids (linoleic, oleic, palmitic, stearic, myristic, elaïdic, lauric, and palmitoleic acids) at similar concentrations that have been identified in human amniotic fluid exerts anxiolytic-like effects similar to diazepam in adult Wistar rats through actions at γ-aminobutyric acid-A (GABA_A) receptors, but unknown is whether any of these fatty acids exerts a predominant action over the others in infant rats. Of these fatty acids, some actions of oleic acid have already been identified, and it is one of the most abundant in amniotic fluid. Therefore, the aim of this study was to explore the effect of oleic acid on anxiety-like behavior and motoric activity in infant rats. To explore sedative actions, 28-day-old Wistar rats received 80-320 µg oleic acid or a sedative dose of diazepam (5 mg/kg). In a dose-response study, other groups of rats were injected with 10-80 µg oleic acid or 1 mg/kg diazepam. In an interaction study, rats that received oleic acid were pretreated with the GABA_A receptor antagonists picrotoxin or flumazenil to explore the participation of this receptor in the effects of oleic acid on behavior in the elevated plus maze, rotarod test, and open field test. Oleic acid produced sedative effects but did not exert any anxiolytic-like actions. Hypoactivity and motor incoordination that were induced by oleic acid were blocked by flumazenil and picrotoxin. In conclusion, oleic acid reduced locomotor activity and motor incoordination through actions at the GABA_A receptor.

Keywords: Hypoactivity, Motor Incoordination, Fatty Acids, Oleic Acid, Anxiety

1. Introduction

Amniotic fluid and colostrum are able to promote useful emotional responses in offspring (e.g., orientation and bonding in lambs through individual odors to ensure survival [1] or the odor of rat dams to learn filial huddling preferences [2]). Human newborns who are exposed to scents from their own amniotic fluid exhibit a reduction of crying when the mother is absent [3]. Apparently, these effects are mediated by fatty acids that are contained in human maternal fluids, which are also able to produce appetitive reactions in human newborns [4] and anxiolytic-like actions that are comparable to diazepam in male and female adult Wistar rats [5, 6]. These effects are blocked by pretreatment with picrotoxin, indicating the participation of γ-aminobutyric acid-A (GABA_A) receptors [7].

GABA_A receptors can be blocked by antagonists, such as picrotoxin and flumazenil. These antagonists block the anxiolytic, sedative, ataxic, and motor incoordination effects of benzodiazepines and barbiturates [8]. Flumazenil is a selective antagonist of the benzodiazepine allosteric binding site, whereas picrotoxin is a noncompetitive antagonist of GABA_A receptors that blocks chloride ion channels [8]. Therefore, these antagonists constitute effective tools in the pharmacological screening of drugs that interact with GABA_A receptors [9].

Oleic acid increases the affinity of flunitrazepam and muscimol, suggesting actions at the benzodiazepine site of
the GABA<sub>A</sub> receptor [10, 11], and modulates the opening of chloride channels [12]. We previously demonstrated that oleic acid is the second most abundant fatty acid in human amniotic fluid, colostrum, and milk [4]. Oleic acid is a monounsaturated fatty acid (C18:1n9) that is widely distributed in nature and the most abundant in such vegetable products as avocados and olives [13, 14]. This may explain the importance of consumption of the Mediterranean diet (i.e., fruits, vegetables, legumes, and nuts), in which wheat and olive oil are the principal fats [15]. The Mediterranean diet apparently produces beneficial effects that maintain health compared with other diets with a low content of these fatty acids [16]. Epidemiological and preclinical studies have suggested some supposedly protective cardiovascular [17], antiinflammatory [18], antioxidant [16, 19], and antitumor [20] effects of oleic acid, but the results have not been conclusive.

Most preclinical studies have focused on identifying anxiolytic and motor effects by employing adult subjects, but anxiety-related behaviors and pharmacological sensitivity can be age-dependent [21]. Anxiety can be detected at early ages [21, 22], but substances with anxiolytic actions in infant rats have been scarcely explored. Therefore, the present study evaluated the effects of oleic acid on anxiety-like behavior and locomotor activity in infant rats. We also explored the participation of GABA<sub>A</sub> receptors in these actions by using the benzodiazepine stearic receptor blocker flumazenil and GABA<sub>A</sub> receptor chloride channel blocker picrotoxin in infant (postnatal day [PND] 28) Wistar rats.

2. Materials and Methods

All of the procedures followed the principles of animal care based on the Guide for the Care and Use of Laboratory Animals [23] and Mexican Official Norm for Care and Use of Laboratory Animals [24]. The experimental protocol received authorization from the Biomedical Research Institute Ethical Committee, National Autonomous University of Mexico (UNAM).

2.1. Animals

The experiments included offspring from Wistar rats, provided by the Biomedical Research Institute, UNAM. During gestation and after delivery, each dam and its litter of 8-10 pups remained in acrylic boxes (44 cm × 33 cm base, 20 cm height) under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) with free access to food (Teklad Lab Animal Diets, Harlan, Indianapolis, IN, USA) and purified water. The nest remained undisturbed, and the dams and pups were minimally handled while changing the bedding material (every 48 h). The offspring were weaned on PND21 and allocated to six to eight animals per cage (44 cm × 33 cm base, 20 cm height). On PND28, the offspring were randomly assigned to the experimental groups and immediately underwent behavioral testing. Not more than three offspring from the same litter were assigned to the same experimental group.

2.2. Experimental Design

2.2.1. Sedative Study

Oleic acid was studied to evaluate its ability to cause sedation. Subcutaneous doses of 80 or 320 µg were chosen based on the oleic acid content in 1 ml of amniotic fluid (80 µg/rat) as the initial point. Two groups of PND28 rats (n = 6 per group) were injected with 80 or 320 µg oleic acid and compared to a group that was injected with 5 mg/kg diazepam (n = 6) as a sedative reference drug and to a vehicle group (n = 6). Two experimental observers evaluated behavior every 5 min for a total of 120 min after the injections. The scoring system was on a 0-4 scale, where 0 = alert and completely responsive, 1 = alert but less active and "wobbly," 2 = awake but drowsy with periods of inactivity or mild sedation, 3 = inactive but readily arousable or moderately sedated, and 4 = unresponsive, unconscious, or deeply sedated [25]. Scores were averaged across treatment groups per time point.

2.2.2. Oleic Acid Dose-Response Study

To explore any anxiolytic-like and motor effects of oleic acid, the rats were assigned to five experimental groups: (i) 10 µg (n = 12), (ii) 20 µg (n = 15), (iii) 40 µg (n = 14), (iv) 60 µg (n = 10), and (v) 80 µg (n = 10), which is equivalent to the amniotic fluid concentration [5]. The data were compared to a vehicle group (n = 10) and 1 mg/kg diazepam group (n = 12) as a reference anxiolytic drug.

2.2.3. Interaction Between Oleic Acid and GABA<sub>A</sub> Receptor Antagonists

Another four experimental groups were included to identify the mechanism of action of oleic acid. The first group received flumazenil (5 mg/kg, n = 9) 30 min before oleic acid administration (80 µg). The second group received picrotoxin (1 mg/kg, n = 10) 30 min before oleic acid administration. These results were compared with an oleic acid group (80 µg, n = 10) and a vehicle group (n = 10). All of the injections were administered subcutaneously in a volume of 1 ml, 1 h before the behavioral tests, with the exception of the antagonists in the interaction study that were dissolved in isotonic saline solution and administered intraperitoneally in a volume of 0.5 ml 30 min before the oleic acid injection.

2.3. Drugs

Oleic acid was prepared according to previous studies at a concentration similar to that found in human amniotic fluid [5], which produces hypoxiaactivity in adult rats [26]. The preparation consisted of oleic acid (8 mg) dissolved in 100 ml of vehicle (96% propylene glycol and 4% ethanol) at a temperature < 40ºC. For the sedative study, 32 mg oleic acid was dissolved in 100 ml of vehicle. Oleic acid, flumazenil, and picrotoxin were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Propylene glycol and ethanol were obtained from J.T. Baker (Xalostoc, Estado de México, México). Diazepam (Valium 10) was obtained from Hoffman-Roche (Basel, Switzerland).
2.4. Behavioral Tests

The rats were first tested in the elevated plus maze and then in the open field test 5 min later. Five minutes after the open field test, they were tested in the rotarod test.

2.4.1. Elevated Plus Maze

The apparatus was constructed of wood and situated in a room with 40 lux illumination. The maze consisted of two opposite open and closed arms set in a plus configuration, elevated 50 cm from the floor. The open arms (50 cm length × 10 cm width) were painted white, and the closed arms (50 cm length × 10 cm width × 40 cm height) were painted black. The rats were individually placed in the center of the maze, facing an open arm. We recorded the number of entries into and time spent on the open arms for 5 min [27]. The anxiety index [28] was calculated: AI = 1 - [(Open arm time / Test duration) + (Open arm entries / Total number of entries)] / 2. The total number of entries was the sum of open arm entries plus closed arm entries. At the end of each individual test, the device was cleaned with a 5% ethanol solution.

2.4.2. Open Field Test

After the elevated plus maze test, each rat was placed in a locomotor activity apparatus for 5 min to evaluate the effects of the treatments on spontaneous locomotor activity. We used an automatic motor activity monitor (Acti-Track v2.7.10, PanLab Instruments, S.L., Barcelona, Spain) constructed of a Perspex box (45 cm × 45 cm) with 35 cm high walls that were elevated 3 cm above the box frame floor. A total of 32 infrared beams, 16 on each perpendicular wall, were mounted on the walls of the box and connected to an interface (LE 8811, LSI Letica Scientific Instruments, Barcelona, Spain) and subsequently to a computer. For data analysis, the floor of the cage was virtually divided into five zones (four peripheral zones and one central zone), and the number of entries into each zone (crossings), total activity time, and total resting time were evaluated for 5 min. No other behaviors, such as rearing or grooming, were evaluated. Because of the relatively small cage dimensions, we did not compare central vs. peripheral exploration.

After each experimental session, the motor activity box was carefully cleaned with a 5% ethanol solution. Five minutes elapsed between each test to allow the scent of the substances to dissipate.

2.4.3. Rotarod Test

To assess the effects of the treatments on motor coordination, all of the rats were trained on the rotarod (LE 8300, LSI Letica, Panlab Scientific Instruments, Barcelona, Spain) for 3 days (PND25, 26, and 27) before the test was conducted on PND28. During the training period, each rat was placed on a horizontal 7-cm diameter rod that rotated at increasing speeds of 4-20 rotations per minute for a maximum period of 5 min to determine baseline performance. The day of the behavioral test, 5 min after the locomotor activity test, the rats were evaluated in the rotarod test. During the test, the rotarod rotated at the same speed at which the rats were previously trained. The test consisted of five trials with a cutoff time of 3 min. The dependent variable was the total time spent (in seconds) on the rotarod. All of the tests were performed between 11:00 AM and 3:00 PM.

2.5. Statistical Analysis

We used two-way repeated-measures analysis of variance (ANOVA) to analyze data from the sedative study, with treatment and time as factors. Significant effects in the ANOVA were followed by the Holm-Sidak post hoc test. A one-way ANOVA was used to analyze the data from the dose-response study and interactions between the effects of the antagonists and oleic acid. Significant effects in the ANOVA were followed by Dunnett’s post hoc test. Values of p ≤ 0.05 were considered statistically significant. In cases of nonparametric distributions of the data, we used the Kruskal-Wallis and Dunn post hoc tests. The results are expressed as mean ± SE.

3. Results

3.1. Sedative Study

The statistical analyses revealed significant effects of treatment (F₁,₄₈₀ = 6.722, p < 0.003) and time (F₇,₄₈₀ = 12.029, p < 0.001) and a treatment × time interaction (F₇,₄₈₀ = 10.968, p < 0.001). The post hoc test showed that 320 µg oleic acid (1.3 ± 0.9) and 5 mg/kg diazepam (1.6 ± 0.8) significantly increased sedation scores compared with the vehicle group (0.1 ± 0.4). The sedative effect of diazepam was evident 5 min after administration and remained up to 60 min (Fig. 1A). Oleic acid at 80 µg (Fig. 1B) and 320 µg (Fig. 1C) produced a longer latency to a sedative effect (i.e., 75 and 80 min post-injection, respectively) compared with vehicle.

![Figure 1. Sedation scores. Diazepam (5 mg/kg) produced a rapid sedative effect (A). Oleic acid at 80 µg (B) and 320 µg (C) produced a longer latency to the sedative effect, regardless of the dose. *p < 0.05, vs. vehicle at the same time of scoring (two-way repeated-measures ANOVA followed by Holm-Sidak post hoc test).](image-url)
3.2. Oleic Acid Dose-Response Study

3.2.1. Elevated Plus Maze

Oleic acid produced similar results as the vehicle group for all of the variables evaluated. As expected, diazepam significantly increased the number of open arm entries (10.5 ± 1.39) compared with vehicle (3.7 ± 0.71; \( H_6 = 27.549, p < 0.001 \)). None of the effects of oleic acid, regardless of dose, were significantly different from vehicle: 10 µg (3.9 ± 0.57), 20 µg (4.8 ± 0.92), 40 µg (4.5 ± 1.05), 60 µg (3.4 ± 0.96), and 80 µg (1.8 ± 0.51).

The statistical analyses revealed differences in the time spent on the open arms (\( H_6 = 25.314, p < 0.001 \)) and the anxiety index (\( H_6 = 23.105, p < 0.001 \)). Differences were found between vehicle (38.8 ± 9.38 s) and diazepam (112.6 ± 11.74 s) but not between the different doses of oleic acid (46.2 ± 9.65 s for 10 µg, 51.3 ± 8.64 s for 20 µg, 42.5 ± 8.63 s for 40 µg, 36.2 ± 12.41 s for 60 µg, and 43.9 ± 27.69 s for 80 µg).

Likewise, the anxiety index was similar between vehicle (0.67 ± 0.04) and 10 µg (0.65 ± 0.04), 20 µg (0.62 ± 0.03), 40 µg (0.69 ± 0.04), 60 µg (0.73 ± 0.06) and 80 µg (0.68 ± 0.13) oleic acid but significantly lower (\( p < 0.05 \)) in rats that were treated with diazepam (0.37 ± 0.05).

3.2.2. Open Field Test

![Figure 2](image_url)

**Figure 2.** Locomotor activity in PND28 rats treated with oleic acid. Oleic acid decreased the number of crossings (A) and activity time (B) and increased resting time (C). Diazepam did not produce any effects on locomotion. *\( p < 0.05 \), vs. vehicle (one-way ANOVA followed by Dunnett’s post hoc test).

Differences in the number of crossings reached statistical significance (\( F_{6,76} = 6.655, p < 0.001 \)). Although no statistically significant dose-response relationship was found, the oleic acid groups presented fewer crossings (\( p < 0.05 \)) compared with the vehicle-treated group (Fig. 2A). Similar results were found with regard to activity time (\( F_{6,76} = 6.338, p < 0.001 \)) and resting time (\( F_{6,76} = 6.342, p < 0.001 \); Fig. 2B, C). Diazepam did not produce effects in the open field test.

3.2.3. Rotarod Test

The treatments dose-dependently affected the total time on the rotarod (\( F_{6,76} = 18.302, p < 0.001 \)). Oleic acid significantly decreased (\( p < 0.05 \)) the total time on the rotarod (Fig. 3).

![Figure 3](image_url)

**Figure 3.** Rotarod results in PND28 rats treated with oleic acid. The total time on the rotarod was lower in the groups that received 60 and 80 µg oleic acid compared with the vehicle group. Diazepam did not produce any effects on motor coordination. *\( p < 0.05 \), vs. vehicle (one-way ANOVA followed by Dunnett’s post hoc test).

3.3. Interaction Between Oleic Acid and GABA<sub>A</sub> Receptor Antagonists

3.3.1. Elevated Plus Maze

No significant differences were found in the number of open arm entries (\( F_{3,35} = 0.684, p = 0.568 \)) between the flumazenil+oleic acid (4.2 ± 0.77), picrotoxin+oleic acid (4.3 ± 0.81), vehicle (4.7 ± 0.44), and oleic acid (3.4 ± 0.58) groups. Likewise, no significant differences in the time spent on the open arms were found between the flumazenil+oleic acid (37.4 ± 7.08 s), picrotoxin+oleic acid (37.7 ± 8.75 s), vehicle (36.6 ± 4.92 s), and oleic acid (39.7 ± 9.99 s) groups (\( F_{3,35} = 0.0276, p = 0.994 \)). Similar results were found for the anxiety index, with no significant differences between the flumazenil+oleic acid (0.75 ± 0.03), picrotoxin+oleic acid (0.78 ± 0.03), vehicle (0.74 ± 0.02), and oleic acid (0.72 ± 0.04) groups (\( F_{3,35} = 0.476, p = 0.701 \)).

3.3.2. Open Field Test

Significant differences in crossings (\( F_{3,35} = 3.297, p < 0.032 \)), activity time (\( F_{3,35} = 4.671, p < 0.008 \)), and resting time (\( F_{3,35} = 4.674, p < 0.008 \)) were found between the oleic acid and vehicle groups, in which oleic acid increased resting time (\( p < 0.05 \)) and decreased crossings (\( p < 0.05 \)) and activity time (\( p < 0.05 \)) by approximately 50% compared with the vehicle group. These effects of oleic acid effect were not observed in rats that were pretreated with
flumazenil and picrotoxin, which were similar to the vehicle group (Fig. 4).

Figure 4. Locomotor activity after GABA_A antagonist administration. The effects of oleic acid on crossings (A), activity time (B), and resting time (C) were blocked by pretreatment with flumazenil and picrotoxin. *p < 0.05, vs. vehicle (one-way ANOVA followed by Dunnett's post hoc test).

3.3.3. Rotarod Test

The total time on the rotarod was significantly affected by treatment (F_{3,35} = 10.453, p < 0.001). The oleic acid-induced decrease in the total time spent on the rotarod was not observed in the flumazenil+oleic acid and picrotoxin+oleic acid groups, which were similar to the vehicle group (Fig. 5).

Figure 5. Rotarod performance after GABA_A receptor antagonist administration. The effect of oleic acid was not observed after flumazenil and picrotoxin pretreatment. *p < 0.05, vs. vehicle (Dunnett’s post hoc test).

4. Discussion

Anxiety-like behavior can be displayed by young rats [29-31]. Research on anxiety in laboratory animals has important implications for the development of new drugs that come from biological sources, such as maternal fluids. After demonstrating that a fatty acid mixture has anxiolytic-like properties in adult rats [5] and that oleic acid is the second most abundant fatty acid in maternal fluids [4], we evaluated different doses of oleic acid on sedation, anxiety-like behavior, and locomotor activity to possibly identify a natural anxiolytic that may be useful for infants. We found that the minimum effective sedative dose was the same concentration that is found in human amniotic fluid. Oleic acid produced a longer latency to a sedative effect compared with diazepam, which produced rapid sedation. Doses of oleic acid that were up to four times higher than the amount found in human amniotic fluid did not produce different sedative effects. Therefore, the amniotic fluid concentration was selected as the maximum dose for the dose-response study to evaluate the anxiolytic-like effects of oleic acid in infant rats.

The elevated plus maze test is widely used to evaluate anxiolytic and anxiogenic drugs [27, 32]. Typically, “anxious” rats spend a shorter time in the open arms and make fewer open arm entries than control animals [33]. Rats that are administered substances with anxiolytic properties, such as benzodiazepines (e.g., diazepam), display more time on the open arms and make more open arm entries compared with controls. In the present study, diazepam increased the time spent on and number of entries into the open arms three-fold and reduced the anxiety index by approximately 50% compared with vehicle. Such effects are considered anxiolytic in adult [5, 27, 28] and infant [34] rats. However, in the present study, oleic acid did not produce any anxiolytic-like effects in the elevated plus maze in infant rats, similar to adult rats [26], indicating its null potency as an anxiolytic agent.

An anxiolytic agent at therapeutic doses ideally must produce the expected response without motor alterations. At an appropriate dose, such substances as alcohol, barbiturates, and benzodiazepines produce anxiolysis. Larger doses produce motor incoordination and sedative actions [35]. In the present study, the main effects of 40-80 µg oleic acid consisted of a reduction of locomotor activity and incoordination. Thus, oleic acid appears to have a pharmacological profile that is similar to drugs with myorelaxant actions [35] and partially shares sedative and hypnotic properties with barbiturates [32]. Reductions of locomotor activity may also be caused by other fatty acids, such as palmitic acid [36] or the amide of oleic acid [37-39]. When polyunsaturated fatty acids were added to a rodent diet, 60% decreases in ambulation, rearing, and exercise time on a running wheel were observed compared with mice that were fed a standard diet [40].

The open field test and rotarod test are two models that present correspondence to different motor activities in rats. In
the open field test, spontaneous ambulation does not involve learning or conditioning and is useful for discarding possible changes in alertness that could interfere with anxiolytic-like behavior [6]. Rotarod performance implies prior training to display coordination and balance on an accelerating rod. The lack of a dose-response effect in the locomotor activity test suggested a floor effect of oleic acid on spontaneous activity, whereas a clear dose-response effect in the rotarod test suggested that oleic acid alters behaviors that are related to coordination and sedation [41].

The anxiolytic and sedative actions of some drugs occur through chloride ion channel opening [12, 42, 43]. Pentobarbital, an anesthetic drug with actions at GABA\(_A\) receptors, produces ataxia and motor incoordination [41], but its potency as an anxiolytic agent is equivocal [44, 45]. A similar profile was found for oleic acid, which increases the affinity of agonists for the benzodiazepine binding site on GABA\(_A\) receptors [10]. Our data suggest that the hypolocomotor effect of oleic acid is mediated by a direct action on GABA\(_A\) receptors because pretreatment with picrotoxin and flumazenil blocked its effects.

Flumazenil [8, 46] and picrotoxin [47, 48] do not have intrinsic actions on anxiety-like or locomotor behavior. Therefore, our results suggest that oleic acid has a direct action on the benzodiazepine binding site of GABA\(_A\) receptors. GABA\(_A\) receptors are composed of \(\alpha\) subunit variants (\(\alpha 1\), \(\alpha 2\), \(\alpha 3\), or \(\alpha 5\)) in combination with a \(\beta\) subunit (\(\beta 1\)-\(\beta 2\)) and \(\gamma\) subunit [49]. Sedation and anxiolysis are modulated by the \(\alpha 1\) and \(\alpha 2\) subunits, respectively [50]. Flumazenil is able to completely prevent the action of zolpidem, a benzodiazepine agonist that acts on the \(\alpha 1\) subunit, to induce motor/turning behavior [51]. In addition to blocking anxiolytic effects, picrotoxin can also reverse ethanol-induced decreases in the righting reflex in rats [52].

5. Conclusion

The present study evaluated the effects of oleic acid that was administered in infant rats on PND28 and explored the participation of GABA\(_A\) receptors in such effects in the elevated plus maze, open field test, and rotarod test. The results showed that oleic acid did not exert any anxiolytic-like actions in the elevated plus maze, but it reduced locomotor activity and motor incoordination apparently through actions at the GABA\(_A\) receptor. In conclusion, oleic acid produced sedative-like effects through GABA\(_A\) receptors without producing anxiolytic-like effects.

Acknowledgements

The authors thank Michael Arends for revising and editing the English of this manuscript. This study was partially supported by a grant from Programa de Fortalecimiento Académico del Posgrado de Alta Calidad (1010/152/2014C-133/2014) from the University of Veracruz, which did not participate in the study design, data collection, data analysis, interpretation of the data, writing of the manuscript, or decision to submit the paper for publication.

J.C.E. and G.G.R. received fellowships from CONACyT (Reg. 197556 and 249708, respectively).

References


