SHORT COMMUNICATION

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Estrogenic response in Japanese turtle (*Mauremys japonica*) exposed to petroleum hydrocarbon

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Abstract

Expressions of the estrogen receptors and vitellogenin in Japanese turtle (*Mauremys japonica*) in response to petroleum hydrocarbon were studied. A total of 15 male turtles were exposed to 1.00 mg L^{-1} of a sample of an oil spill, and 15 male and 15 female turtles were served as controls without an oil spill. The transcripts' results demonstrated an increase over time with greater expression of vitellogenin I in males exposed to petroleum with significant differences. In the case of vitellogenin II, the expression was greater than control males, but it was similar to the values of control females. Concerning the estrogen receptor α and estrogen receptor β, males exposed to oil spill presented higher values at 72 hr than the controls. In conclusion, in the present work, the effect of petroleum as an endocrine disruptor in turtles was demonstrated, and it can be used to identify damages induced by the presence of hydrocarbons in aquatic environments.

Introduction

The exposure to endocrine disruptors (EDCs) of the wildlife is global. The EDCs alter hormone balance, some at low doses and by various mechanisms. Hormone levels vary with age, sex, and time, so to estimate the consequences of EDCs exposure, each species' hormonal pattern should be taken.[1-3] Four major routes of EDCs action can be distinguished: a) Binding to activation of androgenic and estrogenic receptors; b) Blockage of estrogen receptors without activation; c) Modification of hormone metabolism; d) Modification of the hormones receptors number.[4,5] Many environmental chemicals affecting endocrine systems are persistent and bio-accumulative, such as halogenated organic, pesticides, drugs, hydrocarbons, and hydrocarbons derivates.[6,7] To understand the links between exposure and adverse effects, the classification of the molecular bases of EDCs and endogenous estrogen needs to be clarified in the development agencies.[8] A sensitive method detecting the exposure and the effects of EDCs is by monitoring the expression of the genes involved.[9,10]

In this study, quantitative-PCR was used to determine the mRNA involved in the gene expression of vitellogenin (VTG) in Japanese turtles (*Mauremys japonica*) exposed to petroleum hydrocarbon.

Materials and Methods

Experimental design and sampling. Japanese turtles were purchased from Aquapolis Acuario (Campeche, Mexico), maintained in a glass aquarium of 20.00 L with up to 3.00 cm chlorine-free hard water for three weeks[11] and fed with food for reptiles (Wardley, Walmart, USA) *ad libitum* three times a day. All procedures were following the guidelines approved by Biological Ethics Committee of EPOMEX Institute, Campeche University (NOM-019-92 STPS-1993).The pilot hydrocarbon came from an oil spill that occurred in 2007 in the Gulf of Mexico. The chemical analysis indicated the presence of aliphatic hydrocarbons of C14 to C39 (69.96%) and aromatic hydrocarbons (0.524 μg g^{-1}) including acenaphthene, acenaphthylene, anthracene, benzo [k] fluoranthene, fluoranthene, fluorene, indeno [1,2,3-cd] pyrene, and phenanthrene.[12]

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Fifteen male turtles were exposed to 1.00 mg L^{-1} of a sample of the oil spill, and 15 male and 15 female turtles were served as controls without an oil spill. The turtles were placed in hard water. The bioassay was carried out over 72 hr in a static test design according to the criteria of chronic exposure.[11] Every 24 hr, five turtles from each treatment were euthanized to extract the liver. The turtles were euthanized by placing in 500 mL bottles having a lid with a tube adapted to supply carbon dioxide for 5 min. Once the unconsciousness was verified, the animals were decapitated.

mRNA expression analysis. Total RNA was isolated from the liver under the manufacturer´s instructions (GeneJET RNA Purification Kit; Thermo Fisher Scientific, Foster City, USA). The RNA was quantified using NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies LLC, Wilmington, USA), and its quality was assessed by the presence of ribosomal bands in ethidium-bromide stained agarose gels. The RNA was diluted to approximately 1.00 mg mL^{-1} for the reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR was performed according to the manufacturer's instructions (TaqMan Reverse Transcription Reagent; Thermo Fisher Scientific, USA). For the relative quantification of gene expression, quantitative-PCR on StepONE Q-PCR equipment (Applied Biosystems, Foster City, USA) was used. The sequences of the primers are shown in Table 1. The PCR conditions were as follows: Initial denaturation at 94.00 ˚C for 30 sec, hybridization at 60.00 ˚C for 30 sec, and extension at 72.00 ˚C for 60 sec. For quantification, β-actin gene was used as a reference, and $2^{\Delta CT}$ method was used to calculate the expression.[13]

Statistical analysis. Values of parameters were used to detect the interaction between petroleum hydrocarbon and exposure time by two-way ANOVA. If a significant interaction was detected between the main effects, the variable was analyzed using a one-factor ANOVA. If there was a significant difference, F-test was performed using the Statgraphics Centurion X (Statgraphics Technologies, Inc., The Plains, USA).

Results

The transcripts' results showed an increase in the time with greater expression of VTG I in males exposed to petroleum compared to controls with significant differences ($p < 0.05$; Fig. 1). In VTG II, the increase in exposed males was higher than control males, but it was lower than control females (Fig. 1). For the estrogens receptors α and β, they presented higher values at 72 hr compared to the controls ($p < 0.05$; Fig. 2).

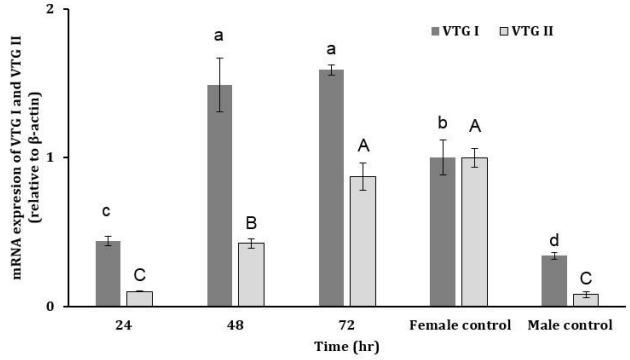

Fig. 1. The relative intensity of vitellogenin I and II (VTG I and VTG II) in the liver of Japanese pond turtle (*M. japonica*). Different letters indicate significant differences at $p < 0.05$.

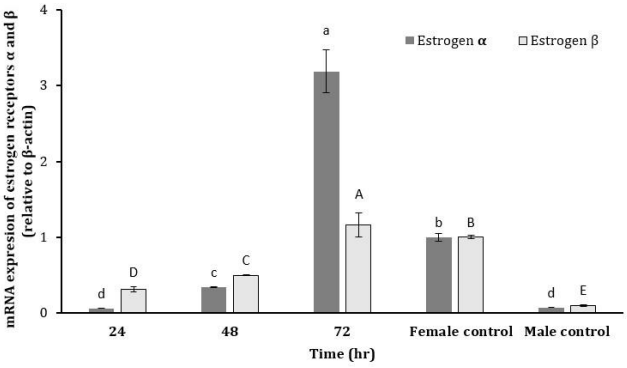

Fig. 2. The relative intensity of estrogen receptors α and β in the liver of Japanese pond turtle (*M. japonica*). Different letters indicate significant differences at $p < 0.05$.

Table 1. Sequences of primer pairs were used in the Q-PCR study.[10]

Gene Name	Primer Sequences
Estrogen receptor α (*AB033491*)	5´-GTCAGTCGGGTTACTTGGCC-3´ 5´-CATCACCTTGTCCCAACCTG-3´
Estrogen receptor β (*AB070901*)	5´-GTGGACTCAACTTTCGGC-3´ 5´-CACGTCGCAGCAGGATCTT-3´
Vitellogenin I (*AB064320*)	5´-TGGAAAGGCTGARGGGGAAG-3´ 5´-AACTGCAGGCATGGTGAGCC-3´
Vitellogenin II (*AB075891*)	5´-GTCTTCAGGAGGTCTTCTTC-3´ 5´-GGTAGACAATGGTATCCGAC-3´
β-Actin (*S74868*)	5´-AGACCACCTACAGCATC-3´ 5´-TCTCCTTCTGCATTCTGTCT-3´

Discussion

In oviparous animals, VTG induction is used as a biomarker to evaluate the presence of estrogenic compounds.[14] In the environment, many contaminating compounds are xenoestrogens and have shown estrogenic response *in vivo* and *in vitro*.[15] In the reptile species, synthesis of estrogen has been observed in other organs aside from ovaries.[16] Some authors have reported a small amount of estrogen production in the adrenal gland and liver, resulting in the local synthesis of VTG.[17] In this study, effects of endocrine disruption on the VTG synthesis were revealed. In studies carried out in sea turtles (*Lepidochelys kempi*), VTG has been detected in adult females blood. The VTG is produced in the liver in the presence of estradiol and deposited in the yolks of eggs.[18] In juveniles and males, the VTG expression is not common, but in the presence of estrogens or xenoestrogens, the expression is possible. Cheek *et al*. have observed the VTG production in juveniles of marine turtles exposed to estradiol.[18] Keller *et al.* have studied 400 loggerhead turtles (*Caretta caretta*), reported an expression of VTG in male and female youth, and concluded that xenoestrogen compounds in the environment might cause this expression.[19] In this study, activations of VTG genes and estrogen receptors were observed over time with oil exposure. Smelker *et al*. have mentioned that the expression of VTG is possible in males in the presence of ECDs in the environment, and it was also verified in the loggerhead turtle (*C. caretta*).[20] Marquez *et al.* have reported that VTG expression in painted turtle (*Chrysemys picta*) males is caused by the presence of petroleum and concluded that hydrocarbons affect the aryl hydrocarbon receptors, a gene regulating the genes involved in the development of gonads.[21] Rochman *et al.* have demonstrated that petroleum alters the endocrine system resulting in a permanent effect on the aquatic fauna.[22]

In conclusion, in this study, the effect of petroleum as an endocrine disruptor in turtles was demonstrated, and it can be used to identify damages induced by the presence of hydrocarbons in the aquatic environment.

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Conflict of interest

The authors declare no conflict of interest.

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