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# Oxytocin neuron activation by acute infusion of *Montanoa* genus plants in the Wistar rats

[Activación de neuronas de oxitocina por la infusión aguda de plantas del género de *Montanoa* en la rata Wistar]

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**Abstract:** In traditional Mexican medicine, plants from the *Montanoa* genus, family Asteraceae (*Montanoa tomentosa*, *Montanoa grandiflora*, and *Montanoa frutescens*) have been used to induce labor owing to their uterotonic properties like those produced by oxytocin (OXT). However, whether infusions of these plants can activate hypothalamic OXT-producing neurons is unknown. To test this possibility, five independent groups of Wistar rats (n=4) were included: intact, vehicle, and three groups that received 50 mg/kg p.o. of *M. tomentosa*, *M. grandiflora*, and *M. frutescens* infusions, respectively. Ninety min after treatment, the brains were obtained and processed using double-labeled immunohistochemistry for Fos protein and oxytocin (Fos/OXT-ir). Rats that received *Montanoa* infusions had significantly greater number of Fos/OXT-ir cells in the paraventricular (PVN) and supraoptic (SON) nuclei, with respect to intact and vehicle groups. These findings demonstrate that *Montanoa* infusions activated OXT neurons, an effect that may be related to the reported pharmacological properties.

**Keywords:** *Montanoa tomentosa*; *Montanoa grandiflora*; *Montanoa frutescens*; Infusions; Oxytocin

**Resumen:** En la medicina tradicional mexicana, plantas del género *Montanoa*, familia Asteraceae (*Montanoa tomentosa*, *Montanoa grandiflora* y *Montanoa frutescens*), se han utilizado para inducir el parto debido a sus propiedades uterotónicas, aparentemente similares a las producidas por la hormona oxitocina (OXT). Sin embargo, se desconoce si las infusiones de estas plantas pueden activar neuronas hipotalámicas productoras de OXT. Para probar esta posibilidad, se incluyeron cinco grupos independientes (n=4): intacto, vehículo y tres grupos que recibieron 50 mg/kg p.o. de infusiones de *M. tomentosa*, *M. grandiflora*, y *M. frutescens*, respectivamente. Noventa minutos después del tratamiento, los cerebros fueron obtenidos y procesados por doble marcaje de inmunohistoquímica para la proteína Fos y oxitocina (Fos/OXT-ir). Las ratas que recibieron infusiones de *Montanoa* aumentaron significativamente el número de células Fos/OXT-ir en los núcleos paraventricular (PVN) y supraóptico (SON), respecto a los grupos intacto y vehículo. Estos hallazgos demuestran que las infusiones de *Montanoa* activan neuronas de OXT, lo que podría estar relacionado con sus propiedades farmacológicas.

**Palabras clave:** *Montanoa tomentosa*; *Montanoa grandiflora*; *Montanoa frutescens*; Infusiones; Oxitocina.

## INTRODUCTION

In traditional Mexican medicine, infusions from three species of the *Montanoa* Cerv., genus*: Montanoa tomentosa* (*Mt*), *Montanoa grandiflora* (*Mg*), and *Montanoa frutescens* (*Mf*), are used to induce labor (Levine *et al.*, 1981; Gallegos, 1985); as contraceptives (Hahn *et al.*, 1981; Gallegos, 1983; Ponce-Monter *et al*., 1983); and as an emmenagogue (Gallegos, 1983; Gallegos, 1985). These pharmacological properties are effectively like those produced by the endogenous oxytocin (OXT) (McNeilly *et al.*, 1983; Stubbs, 2000). Additionally, concoctions from these plants are recommended to alleviate symptoms of depression and anxiety in women (Ximenez, 1615; Levine *et al.*, 1981; Gallegos, 1985). Corroborating this, the three *Montanoa* species (*Mt*, *Mg*, *Mf*) produce anxiolytic-like (Carro-Juarez *et al.*, 2012; Rodríguez-Landa *et al.*, 2014a; Rodríguez-Landa *et al.*, 2014b; Sollozo-Dupont *et al*., 2015) and antidepressant-like effects (Rodriguez-Landa *et al.*, 2018; Lagunes-Merino *et al.*, 2020) in both male and female rats in preclinical studies.

More than 70 phytochemical compounds have been characterized in the genus *Montanoa*; among these, terpenoids (di-, mono-, sesqui-, and triterpenoids) stand out for their uterotonic and contraceptive properties as tested in *Mt*, *Mg*, and *Mf* (Enriquez *et al.*, 1996; Ovaa *et al*., 2001; Villa-Ruano & Lozoya-Gloria 2014). Additionally, flavonoids such as isoquercitrin have been identified in *Montanoa* infusions (Oshima *et al.*, 1986); preclinical studies have reported that isoquercitrin alleviates certain stress-related effects, such as anxiety- and depression-like behaviors (Pathak *et al.*, 2013).

OXT is synthesized by neurons in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. OXT produced from magnocellular neurons in the SON is released into the bloodstream, while OXT produced by parvocellular neurons in the PVN is projected to many areas of the brain (Uvnäs-Moberg, 1998), where it acts at central and peripheral levels to regulate physiological processes such as uterine contractions during labor (Fuchs *et al.*, 1982). Moreover, OXT is involved in maternal (Pedersen *et al.*, 1982), social (Uvnäs-Moberg, 1998), and sexual (Carter, 1992) behaviors, among others, and is released in response to physical and psychological stressors (Gibbs, 1984; Carter & Lightman, 1987; Higuchi *et al.*, 1988; Sanders *et al.*, 1990), by modulating the hypothalamic-pituitary-adrenal axis response (Windle *et al.*, 1997; Neumann, 2002).

Recently, activation of OXT neurons by the infusion of *Mt* in male Wistar rats was reported (Lagunes-Merino *et al*., 2020). However, unknown whether infusions of *Mf* and *Mg* can also induce this effect is unknown. Therefore, this study aimed to explore the effects of acute oral infusions of the three *Montanoa* species on the activation of oxytocinergic neurons in the PVN and SON of the hypothalamus in male Wistar rats. We performed double-labeled immunohistochemistry for OXT and the protein Fos, as an index of cellular activation.

## MATERIALS AND METHODS

### *Ethics*

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Research Council (Publication No. 85-23, Revised 1996) and the Official Mexican Norm for the Care and Use of Laboratory Animals (Official Mexican Norm, NOM-062-ZOO-1999). All efforts were made to minimize animal discomfort during this research in accordance with to the 3R principles (Reduce, Refine, Replace) of preclinical research (Russell *et al.*, 2005).

### *Ethical Approval*

This project was approved by Ethical Internal Committee from Instituto de Ciencias de la Salud, Universidad Veracruzana (CICUAL-ICS, Reg. No. 2019-003)

### *Animals*

Twenty adults male Wistar rats, weighing 300 - 350 g, were included in the study. The rats were housed (4 per cage) in plexiglass containers (33 x 44 x 20 cm) under a 12/12 h light/dark cycle (lights on at 7:00 h), at an average temperature of 25±2ºC, with *ad libitum* access to water and food (Nutricubo Harlan®, S.A. de C.V., Mexico).

### *Preparation of the infusions*

*Mt*, *Mf*, and *Mg* were collected from their natural habitat near Tlaxcala, México. The specimens were authenticated by an expert from the herbarium (TLXM) at the Universidad Autónoma de Tlaxcala (Thiers, 2016), where they were preserved (serial numbers *Mt* UATX10, *Mf* UATX11, and *Mg* UATX12). The botanical authentication in the International Plant Names Index corresponds to *Montanoa tomentosa xanthiifolia* specimen from Kew´s herbarium: K000487579, *Montanoa grandiflora* specimen from Kew´s herbarium: ID

1470964, and *Montanoa frutescens* specimen from Kew´s herbarium: ID K000487574. The leaves were dried for 20 days under the sun in ambient conditions. Once dried, the material was ground into a fine powder with an average weight of 1 g, which was mixed with 20 mL of purified water. The water was heated before its boiling point, removed from the heat, the obtained powder was added, and allowed to cool to room temperature before being filtered. The infusions obtained thus had a concentration of 50 mg/mL. All infusions were prepared 40 min prior to administration, and the dose was calculated according to recommendations for human use as described in traditional medicine.

***Treatments***

Rats were randomly assigned to five independent groups (n = 4 each): intact (Int, no treatment), vehicle (Veh, 1 mL/kg of purified water), *Mt* (50 mg/kg), *Mg* (50 mg/kg), and *Mf* (50 mg/kg) infusions. This number was based on previous studies in which four rats per group were sufficient for immunohistochemical analysis, (Caba *et al.,* 2003; Lagunes-Merino *et al.* 2020) and detecting neuronal immunoreactivity without compromising the statistical power. Therefore, the low number of rats per group adheres to the 3R principles of preclinical research (Russell *et al.,* 2005).

The extracts were administered *per os* (*p.o*.) at a volume of 1 mL/kg, using a gavage stainless steel curved cannula (18G X 3.0" w/2.5 mm ball; Cadence, Inc., Staunton, VA, USA) coupled to a 1-mL disposable syringe (Terumo Medical de Mexico, S.A. de C.V., Mexico City, Mexico). After 90 min of the administration, rats were perfused to obtain their brains.

***Perfusion***

Rats were euthanized with an overdose (*i.p*.) of sodium pentobarbital (Pisa Agropecuaria, S.A. de C.V., Atitalaquia, Hidalgo, Mexico), subsequently perfused intracardially with a 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). Their brains were removed immediately after perfusion and cryoprotected successively in 10, 20, and 30% sucrose solutions. Subsequently, 50-µm coronal sections were obtained in a cryostat (Leica CM1520) at -23°C. Serial sections were collected from the rostral border of the preoptic area to the rostral border of the mammillary bodies and placed in 0.1 M PB until the immunohistochemical assays.

***Immunohistochemistry***

Fos and OXT immunohistochemistry in free flotation was performed according to the protocol outlined in a previous study (Caba *et al.,* 2003). Brain slices were washed several times with 0.1 M PB. Sections were incubated in 0.5% hydrogen peroxide to remove endogenous peroxidases, then incubated in primary antibody against Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 1:5000, and diluted in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in 0.1 M PB and 3% normal horse serum for 72 h. Subsequently, slices were incubated in a biotinylated secondary anti-goat antibody made on horse (Vectastain® Vector Labs, Burlingame, CA, USA) at a concentration of 1:200 for 1 h. Upon completion, the avidin-biotin-HRP complex (Vectastain® Vector Labs, Burlingame, CA, USA) was incubated at a concentration of 1:250. After each incubation, the tissue was washed with 0.1 M PB. Labeling was revealed with 0.05% diaminobenzidine (Polyscience, Warrington, PA, USA) in the presence of 10 mg/mL nickel sulfate (Fisher Scientific, Pittsburgh, PA, USA), 10 mg/mL cobalt chloride (Fischer Scientific, Pittsburgh, PA, USA), and 0.01% hydrogen peroxide, which produced a purple precipitate in the cell nucleus. Subsequently, to obtain double-labeling, the sections were incubated again, this time for 48 h in 0.3% PBT with a primary antibody against OXT (1:5000; Millipore Corporation, Billerica, MA, USA). Sections were then incubated in a biotinylated anti-mouse secondary antibody (1:200, Vectastain® Vector Labs, Burlingame, CA, USA). Finally, the tissue was exposed to a biotinylated anti-mouse secondary antibody, and revealed with 0.05% diaminobenzidine and 0.01% hydrogen peroxide, which produced a brown precipitate in the cytoplasm. The tissue was mounted on gelatinized slides, dehydrated, and covered with a slide with Permount (Fisher Scientific, NJ, USA) for analysis.

***Tissue analysis***

The anatomical locations of the PVN and SON were determined according to the Paxinos and Watson's rat atlas (Paxinos & Watson, 2007). The slides were analyzed under an optical microscope (Leica DM100 LED/DFC450C) at 20X magnification, and the immunoreactive cells were counted manually by two independent observers until >95% agreement was reached. Counting was performed unilaterally in the hypothalamic PVN and SON. For this analysis, the number of Fos/OXT-ir expressing cells was counted in both nuclei at the level of bregma, -0.60–-1.92

mm. Fos-ir was identified by black nuclear staining, and OXT-ir as a brown precipitate in the cytoplasm. The double-labeled neurons had black nuclei and brown cytoplasm.

### *Statistical analysis*

Data were analyzed using a one-way ANOVA, for independent groups with a Dunnett's *post hoc* test applied (SigmaStat 3.5), considering a $p<0.05$ as statistically significant. The data were presented as Means±Standard error.

## RESULTS

The statistical analysis revealed that the total number of OXT cells in the PVN was comparable across the five groups, $F_{(4,15)} = 1.555$; $p=0.237$. However, 90 min after each *Montanoa* administration, some Fos/OXT-ir cells were activated: 6.40% for *Mt*, 7.23% for *Mg*, and 6.16% for *Mf*. With respect to the total number of OXT cells in the SON, no significant differences among treatment groups were found, $F_{(4,15)} = 0.662$; $p=0.628$. However, 90 min after each *Montanoa* administration, some Fos/OXT-ir neurons were activated: 3.91% for *Mt*, 3.25% for *Mg*, and 5.37% for *Mf* (Table No. 1).

**Table No. 1**
**Mean number (± SE) of OXT and double-labeled Fos/OXT-ir neurons in the PVN and SON in the different treatment groups**

| Nucleous PVN | Condition | Total Neurons OXT-ir me ± s.e. | Double labeled Fos/OXT-ir me ± s.e. | % double labeled |
|---|---|---|---|---|
| | *Mt* | 164.5 ± 5.54 | 10.5 ± 0.64 | 6.40 |
| | *Mg* | 141.75 ± 4.38 | 10.25 ± 1.03 | 7.23 |
| | *Mf* | 146 ± 8.78 | 9.0 ± 1.08 | 6.16 |
| | Vh | 149.75 ± 12.13 | 0.5 ± 0.28 | 0.03 |
| | Int | 139.25 ± 6.54 | 0 | 0 |
| | | | | |
| **SON** | | | | |
| | *Mt* | 95.75 ± 6.26 | 3.75 ± 1.43 | 3.91 |
| | *Mg* | 92.25 ± 10.81 | 3.0 ± 1.22 | 3.25 |
| | *Mf* | 102.25 ± 9.73 | 5.5 ± 1.55 | 5.37 |
| | Vh | 92.25 ± 8.29 | 0.5 ± 0.05 | 0.54 |
| | Int | 79.75 ± 6.14 | 0 | 0 |

Statistical analysis indicated a significant difference among treatments, $F_{(4,15)} = 52.008$; $p<0.001$, in the number of double-labeled Fos/OXT neurons in the PVN. The *post hoc* test showed a significant increase in the number of Fos/OXT-ir neurons in the *Mt*, *Mg*, and *Mf* infusion-treated rats, compared to those in the intact and vehicle-treated groups (Figure No. 1). Figure No. 2 shows a representative micrograph of sections with double-labeled Fos/OXT-ir cells in the PVN.

The statistical analysis of the SON revealed a significant difference, $F_{(4,15)} =7.299$; $p=0.002$, in the number of Fos/OXT-ir neurons, among treatment group. The *post hoc* test showed that there was a greater number of active OXT neurons in the groups that received infusions of *Mt*, *Mg*, and *Mf* compared to the intact and vehicle groups (Figure No. 3). Figure No. 4 shows a micrograph of representative sections with double-labeled Fos/OXT-ir cells in the SON.

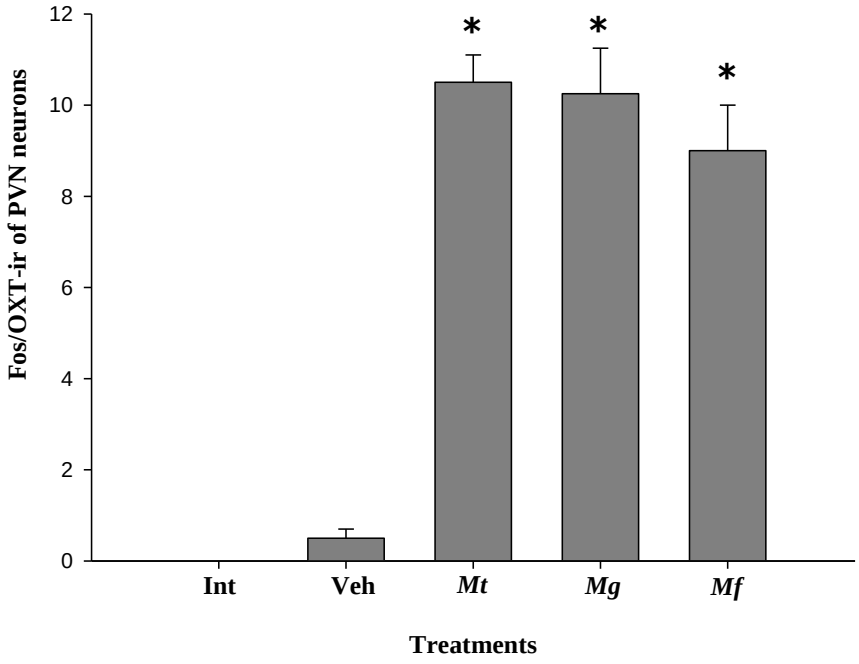


**Figure No. 1**
**Number of double-labeled Fos/OXT-ir cells in the PVN across treatment groups. One-way ANOVA for independent groups, Dunnett's *post hoc* test. *Montanoa tomentosa* (*Mt*), *Montanoa grandiflora* (*Mg*), *Montanoa frutescens* (*Mf*), intact (Int), vehicle (Veh). **p*=0.001**

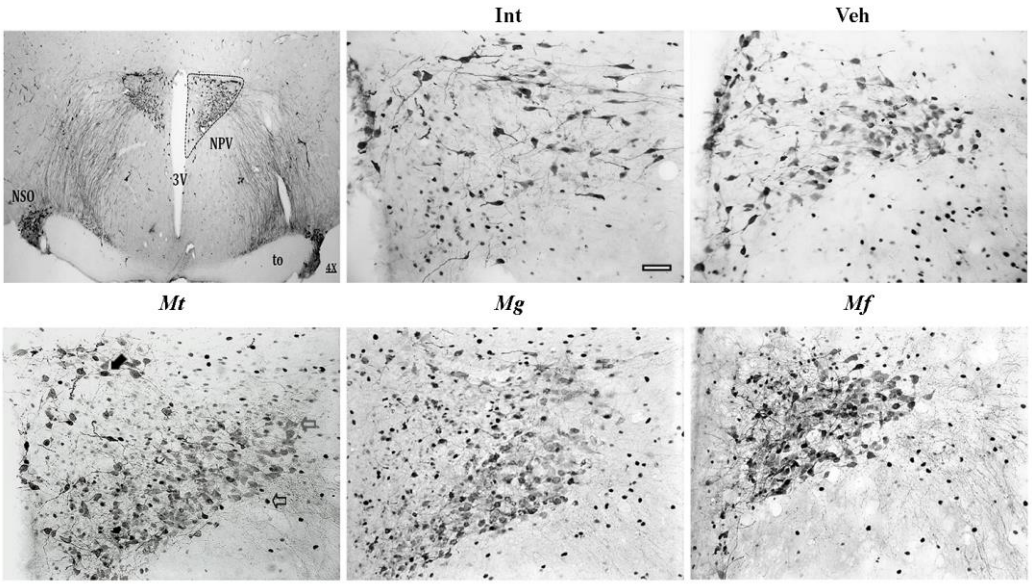


**Figure No. 2**
**Micrograph of representative sections with double-labeled Fos/OXT-ir cells in the middle portion of the PVN, illustrating Fos (empty black arrow), OXT (empty gray arrow), and Fos/OXT (filled black arrow) expression according to treatment group. Bar calibration is 50 µm, objective 20X magnification**

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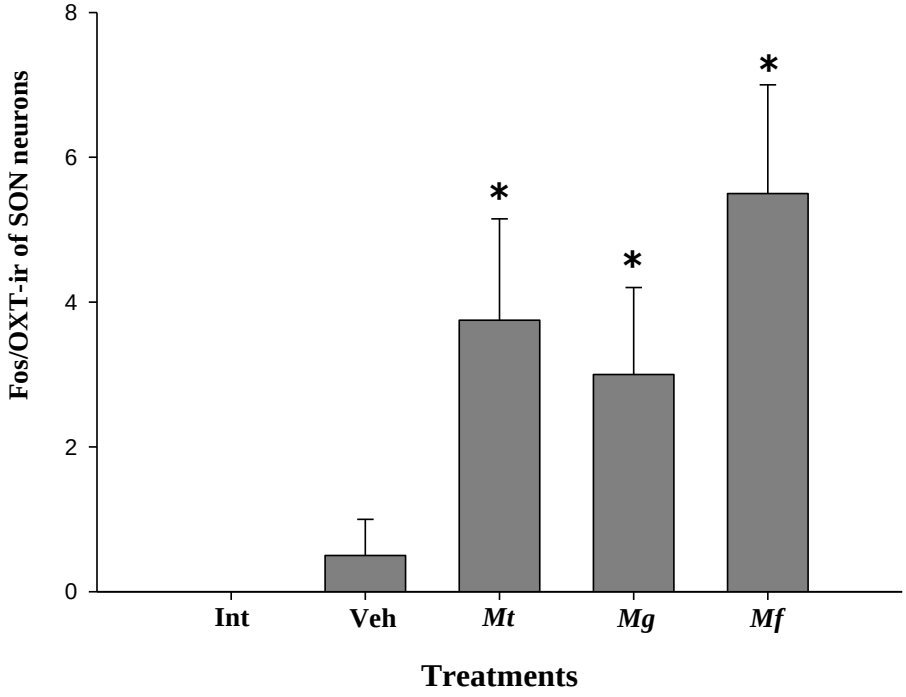


**Figure No. 3**
**Number of double-labeled Fos/OXT-ir cells in the SON according to the treatment groups. One-way ANOVA for independent groups with Dunnett's *post hoc* test. *Montanoa tomentosa* (*Mt*), *Montanoa grandiflora* (*Mg*), *Montanoa frutescens* (*Mf*), intact (Int), vehicle (Vh). **p*=0.002**

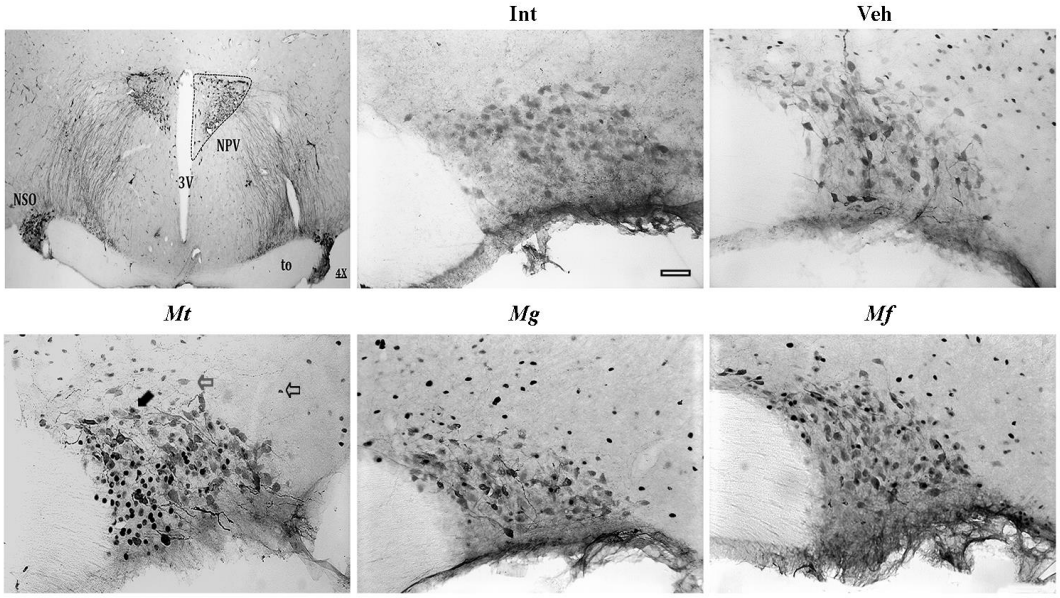


**Figure No. 4**
**Micrograph of representative sections with double-labeled Fos/OXT-ir cells in the middle of the SON, illustrating Fos (empty black arrow), OXT (empty gray arrow), and Fos/OXT (filled black arrow) expression according to treatment group. Bar calibration is 50 µm, objective 20X magnification**

## DISCUSSION

The present study elucidates that acute oral administration of *Mt*, *Mg*, and *Mf* infusions increases the number of active OXT-producing neurons in both the PVN and SON; this activation may be associated with the therapeutic properties attributed to these plants in ancient traditional Mexican medicine and scientific literature.

Neuroscientific studies have shown that neurons transiently manifest early expression genes, such as the proto-oncogene *c-Fos*, which is a promoter of the Fos nuclear protein (Hoffman *et al.*, 1993). Activation of this gene by several stimuli increases Fos protein expression, which is useful as an indicator of neuronal activity that is used to identify neurons and neuronal networks in diverse areas of the nervous system (Hoffman & Lyo, 2002). The maximum expression of Fos protein in the brain tissue occurs between 90 and 120 min after the stimulus administration (Bisler *et al.*, 2002). In the present study, an increased number of Fos/OXT-ir neurons in the PVN and SON was identified 90 min after administering the *Mt*, *Mg*, and *Mf* infusions, suggesting an oxytocinergic activation in these hypothalamic nuclei. This activation of hypothalamic OXT neurons indicates that some secondary metabolites contained in the *Montanoa* infusions interact with the oxytocinergic system. Although we did not perform a phytochemical profile of the *Montanoa* infusions, flavonoids such as isoquercitrin contained in the *Mt, Mf, and Mg* extracts (Oshima *et al.*, 1986) may be involved in the oxytocinergic activation. This hypothesis is supported by the evidence that isoquercitrin exerts uterotonic activity (Bejar *et al.*, 2000), which is modulated by OXT (Shmygol *et al.*, 2006). Recently, some flavonoids and isoflavones were determined to facilitate the release of OXT, similar to some neurosteroids, through mechanisms that involve gamma-aminobutyric acid ($GABA_A$) receptors (Dekermendjian *et al.*, 1999; Wasowski & Marder, 2012). $GABA_A$ receptors possess binding sites for compounds such as benzodiazepines (BZDs) and certain steroids (Sieghart, 1995). Importantly, some natural flavonoids and synthetic derivatives also bind with high affinity to the BZD receptor (Medina *et al.*, 1998; Marder *et al.*, 2003). Additionally, flavonoids contained in *Mt* infusion purportedly activate the GABAergic transmission in parallelly increasing the OXT neuron activity (Lagunes-Merino et al., 2020), which supports the effect reported here.

Although GABA is the main inhibitory neurotransmitter in the central nervous system (CNS), Widmer *et al.* (2003) reported that it can depolarize oxytocin cells, because administration of muscimol ($GABA_A$ receptor-agonist) in the SON evokes OXT release. We observed that an acute administration of the *Montanoa* infusions caused activation of the OXT neurons in the SON, suggesting that the flavonoids in the infusions could be responsible for this activation. The possible action of isoquercitrin and other infusion metabolites on OXT release needs to be further explored.

Other candidate substances in these *Montanoa* species are diterpenes (Kanojia *et al.*, 1982; Levine *et al.*, 1979), which have oxytocic properties. Among these substances, kauradienoic acid from *Mt*, and kaurenoic acid from *Mf* (Campos-Bedolla *et al.*, 1997) are known to facilitate labor (Lozoya *et al.*, 1983), as they trigger utero-relaxing effects (Enriquez *et al.*, 1984). Kaurenoic acid was recently determined to cross the blood-brain barrier and exert its action on the CNS through $GABA_A$ receptors via the chlorine channel complex (Okoye *et al.*, 2013). Therefore, these diterpenes may also be involved in the OXT activation identified in the present study. However, further research is needed to identify the specific mechanism of action that produces this pharmacological effect.

## CONCLUSION

Infusions of *Mt*, *Mg*, and *Mf* activate OXT neurons in the PVN and SON. This effect may be related to the oxytocinergic properties of the *Montanoa* species described in traditional Mexican medicine.

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