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Ethanol, Acetaldehyde and Estradiol Affect Growth and Differentiation of Rhesus Monkey Embryonic Stem Cells

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Abstract

Background—The timing of the origins of fetal alcohol syndrome have been difficult to determine, in part because of the challenge associated with in vivo studies of the peri-implantation stage of embryonic development. Because embryonic stem cells (ESCs) are derived from blastocyst stage embryos, they are used as a model for early embryo development.

Methods—Rhesus monkey ESC lines (ORMES-6 and -7) were treated with 0, 0.01%, 0.1%, or 1.0% ethanol, 1.0% ethanol with estradiol or 0.00025% acetaldehyde with or without estradiol for 4 weeks.

Results—Although control ESCs remained unchanged, abnormal morphology of ESCs in the ethanol and acetaldehyde treatment groups was observed before 2 weeks of treatment. Immunofluorescence staining of key pluripotency markers (TRA-1- 81 and alkaline phosphatase) indicated a loss of ESC pluripotency in the 1.0% ethanol group. ORMES-7 was more sensitive to effects of ethanol than ORMES-6.

Conclusions—Estradiol appeared to increase sensitivity to ethanol in the ORMES-6 and -7 cell line. The morphological changes and labeling for pluripotency, proliferation and apoptosis demonstrated that ethanol affects how these early cells develop in culture, their differentiation state in particular. The effects of ethanol may be mediated in part through metabolic pathways regulating acetaldehyde formation, and while potentially accentuated by estradiol in some individuals, how remains to be **determined**.

Keywords

Primate; fetal alcohol syndrome; embryo development

Introduction

Prenatal ethanol exposure is widely associated with the development of fetal alcohol syndrome (FAS) that affects approximately 0.5-2.0 per 1,000 births (May and Gossage, 2001). Avoiding alcohol consumption during pregnancy will prevent FAS, however, by the time pregnancies are confirmed, major embryonic events may have already occurred (Smith, 1997). Case reports that document FAS pregnancies in women that drink ethanol only

during the first trimester or only until a positive pregnancy test (e.g. Itthagarun et al., 2007) support the hypothesis that the early stages of pregnancy are significantly affected by alcohol consumption.

Acetaldehyde is a major metabolic product of ethanol. The levels of acetaldehyde in circulation are approximately 10-fold lower than the levels of ethanol, due to the NAD-linked alcohol dehydrogenase that permits reduction of acetaldehyde back to ethanol (Deitrich et al., 2007; Umulis et al., 2005). However, the acetaldehyde is reformed and is only removed from the body by oxidation to acetate by various aldehyde dehydrogenases. Acetaldehyde plays a major role in the pathology of ethanol by forming protein adducts that are found in the blood after heavy drinking and are used as a biomarker of alcohol abuse (Hannuksela et al., 2007; Niemela, 2007). Acetaldehyde adduct deposition in many tissues has been demonstrated in humans and animals (Niemela, 2007). To better separate the direct effects of ethanol from those of acetaldehyde, this study is designed to test the effects of both compounds.

The metabolic pathways of estrogen may also impact on the metabolism of ethanol. Interestingly, there is evidence that women are more susceptible than men to alcohol induced liver damage (Corrao et al., 1997). Women have higher acetaldehyde levels when intoxicated during high the estrogen phase of the menstrual cycle (Eriksson et al., 1996). Also, in rats, estrogen increases early alcohol induced liver damage (Yin et al., 2000).

The timing of the origins of FAS have been difficult to determine, in part because of the challenge associated with in vivo studies of the peri-implantation stage of embryonic development. Because embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts and retain a high degree of pluripotency, they are used as an in vitro model for early embryonic development. ESCs as a model for toxicology studies is still relatively recent, however, they have been validated for early embryonic toxicological studies by the European Centre for the Validation of Alternative Methods (ECVAM) (Genschow et al., 2004; Pellizzer et al., 2005). Although murine ESCs have been used as a model for ethanol research (Adler et al., 2006; Arzumanyan et al., 2009), a mouse model for embryonic development may not be ideal. The nonhuman primate is well established as model for developmental toxicology (Hendrickx et al., 1998, 2000) and is superior to rodent models, in part because of the similarity to humans in endocrine control of pregnancy (Elger, 2000).

Rhesus macaque ESC (rESC) lines are more similar to human ESCs than are murine ESCs. Mouse ESCs differ from primate ESCs in cell and colony morphology, growth requirements and molecular markers of pluripotency and differentiation (Pau and Wolf, 2004). For example, leukocyte inhibitory factor (LIF) is able to maintain undifferentiated murine ESCs but not human or non-human primate ESCs (Pau and Wolf, 2004). Another example is that for primate ESCs, the molecular marker SSEA1 indicates differentiation, while in murine ESCs it is a marker of pluripotency maintenance (Henderson et al., 2002). Therefore, this study utilizes rhesus ESCs to evaluate the potential influences of ethanol and its major metabolite on growth and maintenance of ESCs as a model for early embryonic development.

Materials and Methods

Cell Culture

Rhesus monkey (*Macaca mulatta*) ESC lines were obtained from the Oregon National Primate Research Center, Beaverton, OR. Oregon Rhesus Macaque Embryonic Stem (ORMES) -6 and -7 lines were maintained on feeder layers of mouse embryonic fibroblast (mEF) cells that had been mitotically inactivated. Cell lines were maintained in Dulbecco's

Modified Eagle/F-12 medium (DMEM/F12, 11320-033, Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 1% minimum essential medium (MEM) non-essential amino acids, 1mM L-glutamine, 0.1mM β -mercaptoethanol, and 0.1% gentamycin in 60mm cell culture dishes (Mitalipov et al., 2006). Cultures were incubated at 37° C in 5% CO₂ and 5% O₂. Components for cell culture medium were purchased from Sigma (St. Louis, MO) unless otherwise noted. All experiments and analyses were repeated 5 times for each cell line with colonies that were cultured in separate dishes so that N=5 for each analysis for ORMES-6 and -7.

Approximately 40 random colonies per dish for both ORMES-6 and -7 were transferred to fresh feeder layers each time ESC colonies were passaged, which occurred every 3 to 4 days for ORMES-6 and every 7 days for ORMES-7. Colonies for passage were taken from a pre-marked, wedge-shaped section of each dish to assure a random sample. Approximately 18 to 24 hours after the initial passage, cells were cultured in DMEM/F12 medium (modified as above) with 0, 0.01%, 0.1%, or 1.0% (v/v) ethanol and with or without 150 pg/ml estradiol (Sigma E2758). Media changes were performed daily and experimental treatments continued for a total of 5 weeks.

To determine the level of ethanol that was sustained between media changes, samples of culture media were taken at 0, 12 and 24 hours from dishes with only MEF cells and with both MEF and ORMES cells. Samples were stored frozen until assayed by a gas chromatography volatile screen (University of California Medical Center Clinical Laboratory). For all treatment levels, ethanol decreased in a linear manner to 40% of the initial dose by 24 hrs of culture.

Colony number and differential morphology counts were assessed after 4 weeks of ethanol treatment by manual scoring of colonies. Imaging of representative colony morphology types was performed using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) and MicroPublisher camera (QImaging, Burnaby, BC). A repeated measures ANOVA with a Tukey's post-test using Prism 4 for Mac (GraphPad software, Inc., La Jolla, CA) to determine differences among treatments.

Immunofluorescence

Standard stem cell markers for all colonies were assessed by immunofluorescence after 4 weeks of culture in ethanol-containing medium. At 4 weeks, 24 colonies from each 60 mm plate were harvested as described above for passaging; three colonies were put in each well of 8-well slides (Nalge Nunc International, Rochester, NY). The slides were maintained under the same conditions and treatments as the corresponding 60mm dishes for 3 days before being fixed with 4% paraformaldehyde. The fixed slides were stored filled with Dulbecco's phosphate buffered saline (DPBS) at 4°C until stained. All staining incubations were for 40 minutes at room temperature. As a control, one well of each slide was excluded from immunofluorescence staining and was maintained in 100 mM 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS; Fisher Scientific, Fair Lawn, NY), pH 8.2, until the end of the staining procedure. Wells for immunofluorescence labeling were permeabilized with 0.2% Triton X-100, 0.1% Tween-20 in Dulbecco's phosphate-buffered saline (DPBS, Sigma), then blocked with 2% normal goat serum (NGS; Sigma G6767) in DPBS with 0.05% Tween-20 (T-DPBS). Primary antibodies for OCT-3/4, SSEA-4, SSEA-1, TRA-1-60 and TRA-1-81 (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:100 in T-DPBS then applied to the appropriate wells. Cy3-conjugated secondary antibodies to mouse IgG and IgM (Jackson Laboratories, Bar Harbor, ME) were diluted 1:50 in T-DPBS and applied to the appropriate wells after washing 3 times with T-DPBS. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) added to the diluted secondary antibodies. During the secondary antibody incubation, the reserved well of each slide was stained using

an alkaline phosphatase detection kit (SCR004, Millipore, Billerica, MA). After a final washing with DPBS, the slides were mounted with FluoroGel (Electron Microscopy Sciences, Hatfield, PA) and sealed with nail polish. Imaging was performed using a Delta Vision microscope (Applied Precision, Issaquah, WA).

Colony Area Measurements

Colony size was measured at 4 weeks after the start of treatment. Images of stem cell colonies were taken at 72 hours after passage through the ocular of a Nikon SMZ645 stereomicroscope (Nikon, Tokyo, Japan) using a digital camera (Kodak model # Z712, Rochester, NY). The area of each colony in the image was determined with ImageJ (NIH, Bethesda, MD). A repeated measures ANOVA with a Tukey's post-test using Prism 4 for Mac (GraphPad software, Inc., La Jolla, CA) to determine differences among treatments.

Labeling of fixed, intact ESC colonies with aberrant morphology

The colonies with aberrant morphology appeared very dense and thick, which can be problematic when trying to determine the cellular structure inside of these colonies. The cells that were inside of these large colonies were investigated by evaluating Oct3/4 staining as well as proliferation (EdU) and apoptosis (active caspase 3) on fixed aberrant colonies. At 4 weeks of ethanol treatment, additional morphologically aberrant colonies from the 1.0% ethanol treatment plate for ORMES -6 and -7 were harvested as above and were put onto a Cell Well insert (Nunc # 137443, Thermo Fisher, Rochester, NY) that had been seeded 24 hours in advance with Mitomycin C treated mEF cells. The medium both above and below the insert was changed daily for 3 days before treatment with EdU reagent (C10337, Invitrogen, Carlsbad, CA) prepared per the kit instructions. The culture inserts were incubated with the EdU reagent for 1 hour, fixed with 4% paraformaldehyde in PBS for 20 min and stored at 4°C in PBS until embedding. Membranes were removed from the inserts with a scalpel, processed and embedded in paraffin blocks for sectioning. 5µm sections were cut, placed on SuperFrost slides (Fisher Scientific, Pittsburg, PA) and dried overnight at 37°C. After deparaffinization, the slides were incubated in 10 mM sodium citrate at 95°C for 40 minutes and allowed to cool in the same solution to room temperature. The slides were transferred to 3% BSA in DPBS and held at 4°C overnight before EdU labeling per the kit protocol. After EdU labeling, the slides were washed with 3 changes of DPBS with 0.05% Tween 20 (DPBS/T) and then blocked for 40 minutes at room temperature in 10% NGS in DPBS/T. The individual sections on each slide were circled with a PAP Pen (Ted Pella, Redding, CA) placed in a humid chamber and incubated for 40 minutes at room temperature with polyclonal rabbit anti-active Caspase3 (AbCam, Cambridge, MA) at a 1:100 dilution in DPBS/T with 2% NGS. Slides were washed $\times$ 3 with DPBS/T and incubated 40 min with 1:100 Alexa635-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) and 2 µg/ml DAPI in DPBS/T with 2% NGS. After the final incubation the slides were washed as before and mounted in FluoroGel (Electron Microscopy Sciences, Hatfield, PA). A different set of colony sections were labeled with monoclonal mouse anti-Oct3/4 instead of EdU, but also labeled with Caspase 3 and DAPI as described above. Images were captured using the DeltaVision Restoration Microscopy System (Applied Precision, Issaquah, WA) equipped with a 20x/0.70 water immersion lens and analyzed with PhotoShop (Adobe Systems, San Jose, CA).

Results

Morphological differences in treatment

ORMES-6 and -7 control group colonies had morphology that is typical of primate ESC colonies, including cells with a high nuclear to cytoplasmic ratio and prominent nucleoli. The colonies were circular with a distinct edge and consistent cell density and thickness

(Figure 1 A, B). The colony average size ($\pm$ S.E.M.) was $4.4 \pm 0.2 \text{ mm}^2$ for ORMES-6 and $3.2 \pm 0.4 \text{ mm}^2$ for ORMES-7 (Figure 2). The treatment groups with higher levels of ethanol as well as the acetaldehyde treatment groups eventually showed an overall decrease in colony size by Week 4 (Figure 2). The average colony size measure included colonies with both normal and aberrant morphology.

After day 7, the ORMES-6 and -7 1.0% ethanol treatment groups had begun to display morphological abnormalities; some colonies had varying densities throughout the colony, with distinct regions of increased opaqueness and markedly irregular borders (Figure 1 C, D). The “footprint” area of these colonies did not increase but an increase in thickness was observed. The percentage of colonies with aberrant morphology is detailed in Figure 3. There was a dose dependent and time-dependent occurrence of aberrant colonies with increased percentages (to a maximum of over 30% in some replicates) in the 1.0% ethanol treatment group at 4 weeks.

The presence of estradiol (E2) in the culture medium altered the percent of aberrant colonies in the 1.0 Ethanol treatment group, especially in the ORMES-7 cell line (Figure 3). Treatment with acetaldehyde, the primary metabolite of ethanol, also produced colonies with abnormal morphology in both ESC lines. However, estradiol did not increase the effect of acetaldehyde.

The percentage of colonies that survived after each passage did not differ from the control in all treatments of both cell lines (data not shown).

Staining of rESC reveals loss of stemness markers with ethanol treatment

The control groups in both rESC lines showed staining consistent for maintenance of stemness throughout the duration of the study (Table 1). Oct-4, SSEA4, TRA-1-60 and TRA-1-81 had positive fluorescence, while SSEA1 was negative. Alkaline phosphatase was positive throughout each colony.

Abnormal staining patterns were primarily seen in the 1.0% ethanol and 1.0% ethanol + estradiol treatment groups in both cell lines (Table 1). Not all colonies in these treatments stained abnormally and Table 1 indicates abnormal staining (bold text) only for treatments in which a majority of replicates exhibited altered staining patterns. SSEA-4, OCT $\frac{3}{4}$, and TRA-1-60 were not affected in a majority of the replicates in all treatment groups. The 1.0 Ethanol group was the only treatment that exhibited decreased TRA-1-81 staining. The only treatments with positive staining for SSEA-1, indicating a loss of stemness, were the 1.0 % ethanol with and without estradiol for ORMES-6 and -7. In contrast, the loss of strong positive labeling for alkaline phosphatase, indicating a loss of stemness, was present at the two lower levels of ethanol for ORMES-7 and the EtOH-E2 and Acetaldehyde treatments for ORMES-6. In general, these results describing a limited loss of stemness did not seem consistent with the high percentage of aberrant colony morphology present in many of the treatment groups (Figures 1, 3).

Proliferation and apoptosis assays do not reveal the true composition of aberrant colonies

Because the loss of typical ESC morphology was not accompanied by loss of stem cell markers, we sought to determine cell fate in these cultures, so assays for cell proliferation and apoptosis were employed. When these assays were performed on disaggregated colonies, there were no differences between any of the treatment groups (data not shown). These data again did not seem consistent with the morphology changes that were clearly apparent in many of the ethanol and acetaldehyde treatment groups in both ORMES-6 and -7 and it was suspected that significant cell loss occurred during the disaggregation with trypsin. Therefore, normal and aberrant intact ESC colonies were fixed, sectioned and

stained for proliferation, apoptosis and pluripotency and the aberrant colonies are shown in Figure 4. The labels for proliferation (EdU, green) and apoptosis (active caspase 3, red) and cell nuclei (blue) are shown in Panel A, while Panel B shows labels for pluripotency (Oct 3/4, green) and apoptosis (red) and cell nuclei (blue). These intact colonies demonstrate that cells with the expected proliferation and pluripotency occur on the surface of the colony, thus explaining why the standard immunofluorescent labeling techniques at times showed normal “stemness” labeling in morphologically abnormal colonies. However, in the under layers of the aberrant colonies with increased density and depth there was a clear disorganization of cellular structure and a high level of apoptosis as well as non-cellular debris.

Discussion

We present here evidence that ethanol disrupts the maintenance of the growth patterns and pluripotency markers of undifferentiated rhesus ESC lines. Furthermore, unlike the effects of ethanol on mouse ESCs (Arzumanyan et al., 2009), ethanol treatment resulted in increased levels of apoptosis and increased colony size under self-renewal conditions. These results underscore the importance of using a primate model for the study of potential effects of substances on human embryonic differentiation. This study also demonstrates that acetaldehyde may be a primary mediator of the effect of ethanol on ESCs and that estradiol may amplify the effects of ethanol in some individuals.

Embryonic stem cells have become a new model to better understand early embryonic development (Dvash and Benvenisty, 2004; Rugg-Gunn et al., 2005; Vallier and Pedersen, 2005). ESCs provide an especially valuable model because the peri-implantation stages of embryo development are the most difficult to study in vivo and these cells are a ready source for evaluating the underlying mechanisms of that ephemeral process. Although human ESC lines are available for study, only nonhuman primate ESC lines can be directly compared to changes that occur in vitro. Ethical concerns prevent any invasive studies of in vivo early human development, therefore, in vitro studies with human ESCs can never be validated through direct comparison with in vivo developmental stages.

Exposure to ethanol early in development (e.g. during gastrulation, a time of cellular rearrangement and establishment of the embryonic germ layers) can result in disruption in growth and lead to FAS (Webster et al., 1980). Critical sensitivity windows for ethanol exposure have not been described for peri-implantation embryos, in part because of the difficulty of studying such events in vivo. The finding that ethanol alters growth and maintenance of rESCs is an indication that peri-implantation embryos are likely targets for in vivo ethanol effects.

The ethanol treatment levels in this study were designed to model for alcohol levels in binge drinking (1.0 %), moderate drinking (0.1%) and chronic low-level alcoholism (0.01%) that have been reported in humans (Heng et al., 2006; National Institute of Alcohol Abuse and Alcoholism, 2004). Although the ethanol levels in the 1.0% group are within binge drinking range, the constant, high level of alcohol exposure is less likely to be an accurate model for the episodic pattern of binge drinking; however, this group is valuable for elucidating differential effects of ethanol that are dose dependent. The ORMES-7 cell line was more sensitive to ethanol than ORMES-6, especially with regard to the appearance of aberrant morphology at a lower level of ethanol exposure (Figure 3). These data support the view that individual variation may be a factor in the development of FAS.

Under control conditions, pluripotency markers such as Oct-4 are present in ESCs and only down-regulated following differentiation (Palmieri et al., 1994). The loss of labeling for

markers of pluripotency in the rhesus ESC colonies under non-differentiation maintenance conditions is similar to the loss of Oct 3/4 previously reported for human ESC lines (Nash et al., 2009). In contrast, Adler et al. (2006) found a transient up-regulation in Oct-4 expression occurred with ethanol treatment of murine ESCs. The difference in response to ethanol between primate and murine ESC lines is not surprising because primate, including human and rhesus, ESC lines are maintained under different conditions than murine ESC lines (Thomson and Marshall 1998). For example, murine cells require LIF and can grow in monolayers and primate cells do not require LIF and grow only in colonies. While in studies of non-ESC cells, ethanol treatment has been associated with a delay in differentiation (Garriga et al., 2000; Kornfehl et al., 1999; Tatenos et al., 2004), a delay in differentiation did not seem to be present in rhesus ESC treated with ethanol. However, further studies on how ethanol affects directed differentiation of primate ESCs may reveal similarities that are not shown in these experiments.

Ethanol induced cellular damage is dependent on a number of factors, and the metabolism of ethanol itself results in several by-products that can have negative impacts on reproduction (reviewed by Brooks, 1997; Eriksson, 2001). The major pathway of ethanol metabolism is alcohol dehydrogenase that results in acetaldehyde production. Ethanol induction of CYP2E1 and associated oxidative stress is also associated with cellular damage, at least in hepatocytes (Lu and Cederbaum, 2008). In addition, lipid peroxidation is significantly increased as a result of the action of ROS and iron on lipids. All of these processes can damage DNA as well as have other direct effects on cells (Brooks, 1997). Protective mechanisms modulate the potential for cellular damage. Heme oxygenases, particularly inducible heme oxygenase type 1 (HO-1), can protect against ethanol-induced cellular injury (Yao et al., 2009), and estrogen and progesterone up-regulate uterine HO-1 expression which is elevated during pregnancy in rats (Cella et al., 2006). It is now known that murine oocytes and pre-implantation embryos express alcohol and aldehyde dehydrogenases (Rout and Armant, 2002). During the time when women may not yet recognize that they are pregnant, but may still be consuming ethanol, oocytes are undergoing final meiotic maturation, fertilization and early embryonic cleavage and implantation. All of these processes require DNA replication and therefore, may be susceptible to perturbation by these ethanol metabolites. Clearly, further studies are needed on potential damaging as well as protective cellular responses to ethanol in early embryonic development.

The metabolic pathways of estrogen may also impact on the metabolism of ethanol. Interestingly, there is evidence that women are more susceptible than men to alcohol induced liver damage (Corrao et al., 1997). Women have higher acetaldehyde levels when intoxicated during the high estrogen phase of the menstrual cycle (Eriksson et al., 1996). Also, in rats, estrogen increases early alcohol induced liver damage (Yin et al., 2000). The potential connections between these metabolic pathways may lead to speculations about mechanisms and further studies.

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Figure 1. Colony morphology

Control colonies (A and B) from ORMES-6 and -7 ESC lines, respectively. Morphologically abnormal colonies (C-D) from ORMES-6 and -7 ESC lines, respectively.

Figure 2. ESC colony morphology after treatment

. The percentage of ORMES-6 and -7 colonies with abnormal morphology after various treatments. Each treatment for each cell line represents an N=5 dishes of separately cultured ESC colonies. Significant differences ($p < 0.0001$) are indicated for ORMES-6 by different superscripts a, b or c and for ORMES-7 by different superscripts x or y ($p < 0.01$).

Figure 3. ESC colony size

Colony area in mm² is presented for ORMES-6 and -7 for each treatment one week after the last passage. Each treatment for each cell line represents an N=5. Significant differences ($p < 0.0001$) are indicated for ORMES-6 by different superscripts a,b or c and for ORMES-7 by different superscripts x or y.

Figure 4. Proliferation, apoptosis and pluripotency labeling of aberrant ESC colonies

Panel A shows labels for proliferation (EdU, green) and apoptosis (Caspase 3, red) and cell nuclei (DAPI, blue) in a rhesus ESC colony with aberrant morphology after treatment with ethanol. Panel B shows pluripotency (Oct 3/4, green) and apoptosis (Caspase 3, red) and cell nuclei (DAPI, blue) in a similar ESC colony.

Table 1

Rhesus embryonic stem cell colony staining for stemness markers after 4 weeks of treatment.

Treatment	Alkaline Phosphatase		SSEA-4		OCT 3/4		TRA-1-60		TRA-1-81		SSEA-1	
	O-6	O-7	O-6	O-7	O-6	O-7	O-6	O-7	O-6	O-7	O-6	O-7
Control	P	P	P	P	P	P	P	P	P	P	N	N
0.01% EtOH	P	WP	P	P	P	P	P	P	P	P	N	N
0.1% EtOH	P	WP	P	P	P	P	P	P	P	P	N	N
1.0% EtOH	P	P	P	P	P	P	P	WP	P	P	WP	WP
0.1% EtOH + E2	P	P	P	P	P	P	P	P	P	P	N	N
1.0% EtOH + E2	WP	P	P	P	P	P	P	P	P	P	WP	WP
0.0025% Acet	WP	P	P	P	P	P	P	P	P	P	N	N
0.0025% Acet + E2	P	P	P	P	P	P	P	P	P	P	N	N

O-6 = ORMES-6; O-7 = ORMES-7; P = positive; WP = weak positive; N = Negative Results outside of normal labeling are in bold letters.