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Anti-oxidant Effect of High Dilutions of *Arnica montana*, *Arsenicum Album*, and *Lachesis Mutus* in Microglial Cells *in Vitro*

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Abstract

Microglial cells play important roles in inflammatory responses. The level of oxidative stress is a well-known marker of inflammation. Homeopathic medicines are often used clinically to alleviate inflammation. We evaluated the anti-oxidative effect of high dilutions of *Arnica montana* (Arnica m.), *Arsenicum album* (Arsenicum a.), and *Lachesis mutus* (Lachesis m.) on production of reactive oxygen species (ROS) in inflamed microglial cells *in vitro*. Microglial cells, on exposure to lipopolysaccharide (LPS), have induced production of ROS compared with resting cells. The dilutions significantly reduced the oxidative stress by decreasing the level of ROS produced. Arnica m. 1C, 3C, 5C, 7C, 9C, and 30C dilutions had a range of ROS reduction between 15 and 42.1%; Arsenicum a. 3C, 5C, 7C, 15C, and 30C dilutions had a range of ROS reduction between 17.6 and 35.3%; and Lachesis m. 3C, 5C, 7C, 9C, 15C, and 30C dilutions had a range of ROS reduction between 25 and 41.7%. To summarize, the dilutions with the greatest effect were Arnica m. 1C (42.1%), Arsenicum a. 30C (35.3%), and Lachesis m. 7C (41.7%). Arnica m., Arsenicum a., and Lachesis m. did not have the same effect on ROS production and were not dose-dependent.

Keywords

inflammation, oxidative stress, reactive oxygen species, high dilutions, *Arnica montana*, *Arsenicum album*, *Lachesis mutus*, microglial cell

Introduction

Inflammation is an essential response of the immune system to a number of harmful stimuli (pathogens, microbial infection, and toxic compounds) thereby ensuring the survival and the protection of cells.[1] The innate immune system consisting of macrophages, microglia (macrophage cells present in the brain), dendritic cells, neutrophils, and lymphocytes plays a key role in inflammatory responses. The microglial inflammatory response involves several mediators such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), both hallmarks of inflammation. Under physiological conditions, different ROS exist in equilibrium. However, in pathological conditions, ROS over-production leads to oxidative stress damaging proteins, nucleic acid, lipids, membranes and organelles. High levels of ROS production can activate various transcription factors including NFκB and STAT and induce inflammation. Homeopathic medicines are often used as an anti-inflammatory in clinical practice. The effect of homeopathic medicine has been studied in experimental inflammation in particular *Arnica montana*, *Arsenicum album,* and *Lachesis mutus*.[2-4]

The whole plant of *Arnica montana* has been widely used in European traditional medicine to treat various pathological conditions, including pain, stiffness, and swelling associated with trauma, post-operative clinical conditions.[5] The main active constituents of this plant are flavonoids and sesquiterpene lactones such as helenalin having an anti-inflammatory effect.[6] The effect of *Arnica montana* 6C

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demonstrated a significant anti-inflammatory effect on carrageenan into rat footpad by reducing the edema intensity.[7]

Arsenicum album (arsenic trioxide) although a well-known toxic heavy metalloid, when used at high dilution as a homeopathic drug, alleviates anxiety, stomach pain, food poisoning, urinary infection, and skin irritation.[8] Arsenic exposure increased oxidative stress level, inflammatory cytokines, and apoptosis.[9] However, high dilutions of *Arsenicum album* improved the inflammation conditions by reducing the oxidative stress markers.[10,11]

Venom of the *Lachesis muta* snake contains enzymes, hemostasis and hemorrhagin toxins, and neurotoxins and cardiovascular toxins. *Lachesis mutus* which is a homeopathic medicine prepared from the venom of the bushmaster snake is used to treat hot flushes, depression and also for diseases with hemorrhagic tendencies. The snake venom induces a local inflammatory edema.[12] However, homeopathic dilutions of *Lachesis mutus* 30C reverse the inflammation condition of hypertensive patients.[13] The effect of *Lachesis mutus* homeopathic dilutions is not well documented.

The anti-inflammatory mechanism of action of these homeopathic dilutions is still unknown. In the present study, we investigated the effect of *Arnica montana*, *Lachesis mutus,* and *Arsenicum album* dilutions on the oxidative stress using a microglial cell model *in vitro*.

Methods

Homeopathic Preparations

Arnica montana (Arnica m.), *Arsenicum album* (Arsenicum a.), and *Lachesis mutus* (Lachesis m.) homeopathic dilutions were prepared by Laboratoires Boiron (Messimy, France) according to the European Pharmacopoeia (Ph. Eur.) guidelines to produce homeopathic remedies (European Pharmacopoeia, Ninth Edition, Supplement 9.4. EDQM, editor. Strasbourg, France: Council of Europe; 2017). Sterile water (OTEC, Aguettant-France) was used as vehicle control. For Arnica m. (from Arnica m. whole plant) and Lachesis m. (from snake venom), the first centesimal (1C) dilution is obtained by dissolving one volume of the mother tincture in ninety-nine volumes of purified sterile water. Then 2C is obtained by mixing one volume of 1C with ninety-nine volumes of purified sterile water. Each dilution is successively subjected to a vigorous agitation called dynamization. For Arnica m., the dilutions tested were 1C, 3C, 5C, 7C, 9C, 15C, 30C, and 200C. For Lachesis m., the dilutions tested were 3C, 5C, 7C, 9C, 15C, 30C, and 200C.

For the preparation of Arsenicum a., the first centesimal (1C) dilution is obtained by trituration using a mortar and pestle with lactose by mixing one part of arsenic trioxide (As_2O_3) with ninety-nine parts of lactose. Then, 2C is obtained by mixing one part of 1C with ninety-nine parts of lactose. Arsenicum a. 3C is obtained by mixing one volume of 2C with ninety-nine volumes of purified sterile water. The next C dilutions are prepared by repeating the same procedure in order to obtain the other subsequent dilutions in the range. The dilutions tested of Arsenicum a. were 3C, 5C, 7C, 9C, 15C, and 30C. All procedures for drug preparation and cell treatments were performed in sterile conditions.

Reagents

Leibovitz's L-15 medium, Dulbecco's Modified Eagle's Medium with low glucose (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and without magnesium, and 0.05% Trypsin-EDTA and antibiotics cocktail 100X (10,000 U/mL penicillin - 10,000 µg/mL streptomycin) were purchased from Gibco. Fetal bovine serum (FBS) French origin was purchased from Biowest. Lipopolysaccharide (LPS) isolated from *Escherichia coli* 0111:B4, Dexamethasone (DEX), Poly(ethyleneimine) solution (PEI), and Formaldehyde solution (37 wt % in H_2O) were all purchased from Sigma-Aldrich-Merck. Cell proliferation Kit II (XTT) was purchased from Roche Applied Science. CellROX Deep Red Reagent was obtained from Invitrogen Life Technologies.

Primary Culture of Microglia From Newborn Mice

Pregnant female C57BL/6J (Charles River, L'Arbresle, France) were housed, handled, and taken care of in AniRa-PBES (Plateau de Biologie Expérimentale de la Souris, Lyon, France) and in accordance with the European Union Council Directives (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments- University Claude Bernard Lyon I (CEEA-055).

Microglial cells were isolated from the brains of postnatal day 0 mouse pups.[14] Briefly, the whole brains were harvested, placed into Leibovitz's L-15 medium, and were mechanically dissociated. After several centrifugation steps, the pellet of brain cells was resuspended in complete culture medium (DMEM with low glucose supplemented with 10% heat inactivated FBS and 1% antibiotics cocktail) and plated on PEI-coated Corning® T-75 flask. After a single medium change for removing cellular debris at day two *in vitro*, the cells were kept for 10 days. Then, some supplemented complete culture medium was added until completion of microglial cell isolation at day 20. The purity of microglial cells was verified at the start of the study by labeling the cells with antibodies, anti-GFAP (glial fibrillary acidic protein, specific of astrocytes) and anti-Iba-1 (ionized calcium binding adaptor molecule 1, specific of microglial cells) and analyzed by flow cytometry. The microglial cells were GFAP negative and Iba1 positive (data not shown).

Oxidative Stress Experiments

The cells were seeded on PEI-coated Nunc® TC Nunclon® 48-well plate at a density of 100,000 cells/well. The next day,

microglial cells were treated with LPS (10 ng/mL) for 24 hours. Treatments with Arnica m., Arsenicum a., and Lachesis m. dilutions (6% v/v), vehicle (6% v/v) were added 4 hours before and 4 hours after initiating the activation with LPS. To inhibit the activation of the cells, the dexamethasone (DEX) which is a glucocorticoid with an anti-inflammatory effect was added at 0.63 µM for 4 hours before LPS activation. All the treatments were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

The level of reactive oxygen species (ROS) was assessed using the cell-permeant CellROX Deep Red Reagent which is a fluorogenic probe. The reagent exhibits bright near-infrared fluorescence upon oxidation that serves as a direct measurement of ROS levels. The microglial cells were stained with 10 µM CellROX Deep Red reagent for 30 min before the end of the treatments, then washed with DPBS, and fixed with 3.7% formalin solution in DPBS before image acquisition. For each well, three images were randomly acquired with identical exposure parameters using an inverted fluorescence microscope (Zeiss, Axio Observer Z1) equipped with Hamamatsu camera (Orca Flash 4.0) and a LD Plan-Neofluar 20x objective. The quantification of fluorescence was performed using open source ImageJ software with a home-made specific plugin developed by Gly-CRRET lab at University Paris-Est Créteil. Results were expressed as a fractional change in fluorescence intensity relative to baseline in untreated cultures without LPS stimulation.

XTT Viability Assay

The viability of the cells was monitored using XTT viability assay. The tetrazolium salt XTT is reduced to an orange soluble formazan by living cells. The amount of formazan directly correlates to the number of metabolically active cells in the culture.

Microglial cells were seeded on PEI-coated Nunc® TC Nunclon® 96-wells plate at the density of 50,000 cells/well. The next day, the cells were treated 4 hours before LPS stimulation with Arnica m., Arsenicum a. and Lachesis m. individual dilutions and vehicle (6% v/v). Then, microglial cells were treated with LPS at 10 ng/mL for 24 hours. At 20 hours after LPS simulation, an XTT labeled mixture was prepared in accordance with manufacturer's instructions and was directly added to each well (final concentration 0.3 mg/mL).

After 4 hours of incubation at 37°C and 5% CO_2, corresponding to 24 hours after LPS stimulation, the absorbance at 465 nm (formazan) and at 650 nm (reference wavelength) were quantified using SPARK 10M microplate reader (Tecan). Absorbance values obtained were used to determine the cell viability. Wells without cells were performed and used as blank reference. LPS-treated cells were considered as viability reference (viability ratio = 1).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 7.00). Each result is representative of at least three independent experiments. Shapiro Wilk normality test was used and comparisons were performed using a non-parametric Kruskal–Wallis test followed by the Dunn's post-hoc test to show significant differences against vehicle with LPS. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

Cell Viability

The microglial cells were incubated with LPS (10 ng/mL) and homeopathic dilutions of Arnica m. 1C, 3C, 5C, 7C, 9C, 15C, 30C, and 200C, Arsenicum a. 3C, 5C, 7C, 9C, 15C, and 30C, and Lachesis m. 3C, 5C, 7C, 9C, 15C, 30C, and 200C. These dilutions did not affect the viability of the cells (Figure 1A, 1B and 1C).

Effect of ROS production with Arnica m., Arsenicum a., and Lachesis m. dilutions

We evaluated the potential anti-oxidant properties of Arnica m., Arsenicum a., and Lachesis m. with a wide range of potencies in LPS-inflamed microglial cells.

When microglial cells were inflamed with LPS (10 ng/mL), the production of ROS significantly increased in a range of 1.6 to 2.3-fold. However, the dexamethasone treatment showed a significant decrease. The addition of the vehicle only did not enhance ROS production compared with LPS. The resting cells (untreated cells) displayed a low level of ROS (Figures 2A, 2B, and 2C; dotted line on the graph equals to one).

Arnica montana. We tested a large range of dilutions of Arnica m. 1C, 3C, 5C, 7C, 9C, 15C, 30C, and 200C on LPS-inflamed microglial cells. The effects of Arnica m. on ROS production are presented in Figure 2A. The results of the treatment of microglial cells with Arnica m. dilutions were consistent and showed a high significant effect on ROS production with the low potencies from 1C to 7C (1.1 to 1.5 *vs* 1.9 vehicle, ****P<0.0001) by reducing ROS level into the cells. Arnica m. 1C seems to have a similar effect on ROS production compared with dexamethasone. Regarding potencies from 9C to 200C, Arnica m. had a significant decrease of ROS production with 9C (1.6 *vs* 1.9 vehicle, *P<0.05) and 30C (1.6 *vs* 1.9 vehicle, **P<0.01) but not a significant effect with 15C and 200C on inflamed microglial cells.

Arsenicum album. The production of ROS (Figure 2B) was measured in LPS-microglial cells with Arsenicum album dilutions. Different potencies of Arsenicum a. 3C, 5C, 7C, and 15C significantly decreased the production of ROS (1.2 to 1.4 (3C, ***P<0.001; 5C, **P<0.01; 7C, *P<0.05) *vs* 1.7 vehicle). The effect of Arsenicum a. 30C is more pronounced (1.1 *vs* 1.7 vehicle, ****P<0.0001), whereas Arsenicum a. 9C

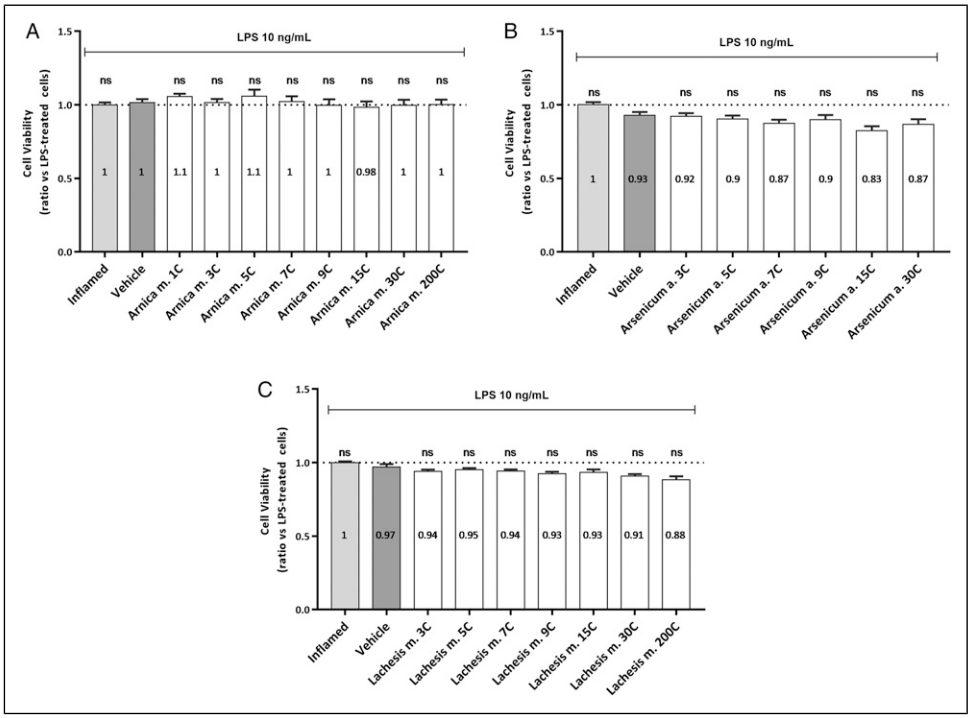

Figure 1. Viability of LPS-inflamed microglial cells after 24 h exposure to homeopathic dilutions of Arnica m. (A), Arsenicum a. (B), Lachesis m. (C), and vehicle. The graph shows the relative cell viability ratio compared with LPS evaluated by XTT assay. Data are represented as mean ± SEM of independent experiments. Inflamed and homeopathic dilutions *vs* vehicle by Kruskal–Wallis test, ns: not significant.

has no significant effect on the production of ROS compared with vehicle treatment. The 3C, 5C, 7C, 15C, and 30C dilutions of Arsenicum a. all have anti-oxidative properties. These dilutions had lower effects compared with the dexamethasone. The effect Arsenicum a. dilutions on ROS production was a non-linear relationship and the response was an inverted U-shaped dose-effect.

Lachesis Mutus. As reported in Figure 2C, the effect of Lachesis m. 3C, 5C, 7C, 9C, 15C, 30C, and 200C dilutions was evaluated. The results showed that Lachesis m. dilutions 3C, 5C, 7C, 9C, 15C, and 30C had a significant diminution of ROS production compared with LPS+vehicle (1.5 to 1.8 Lachesis m. *vs* 2.4 vehicle, ****$P<0.0001$ (3C, 5C, 7C, and 9C) and **$P<0.01$ (15C and 30C)). Interestingly, Lachesis m. 7C dilution seemed to reduce the oxidative stress more efficiently. Noteworthy, this decrease was close to the one observed with the dexamethasone. Only Lachesis m. 200C has no significant effect on the production of ROS compared with vehicle. Overall, most Lachesis m. dilutions tested displayed a significant anti-oxidative activity.

Discussion

The objective of this study was to assess the anti-oxidative effect of homeopathic dilutions of Arnica m., Arsenicum a., and Lachesis m. on mouse microglial cells exposed to LPS (bacterial endotoxin). In this model, LPS initiates inflammation and leads to ROS production. The level of ROS was assessed using the fluorogenic CellROX Deep Red Reagent probe. The effects of a large range of homeopathic dilutions were examined on intracellular ROS generation.

Arnica montana. In LPS-inflamed microglial cells, Arnica m. 1C, 3C, 5C, 7C, 9C, and 30C dilutions significantly decreased ROS production. Arnica m. 1C dilution showed the highest effect by reducing ROS production. This effect on oxidative stress with Arnica m. dilutions that we observed could explain the anti-inflammatory properties and moreover its anti-inflammatory effects as observed in clinical practice.[15] The beneficial effects of *Arnica montana* are well documented. The main active compound responsible for biological effects of *Arnica montana* is the sesquiterpene lactone helenalin. One of the targets of helenalin is the transcription factor NFkB by inhibiting the inflammatory cytokines secretion.[16] Helenalin (around 0.5-1 µM) incubated with THP-1, Jurkat cells, and PBMC induced a cell growth inhibition because the helenalin showed a cytotoxicity. This compound on cells enhanced the formation on intracellular ROS.[17] However, the homeopathic dilutions of *Arnica montana* 30C on *E. coli* exposed to UV showed less DNA damage and less oxidative stress by decreasing ROS generation and increasing in SOD, CAT and GSH activities.[18] Moreover, *Arnica montana* 6C was investigated by Macedo *et al.* on carrageenan and rat paw

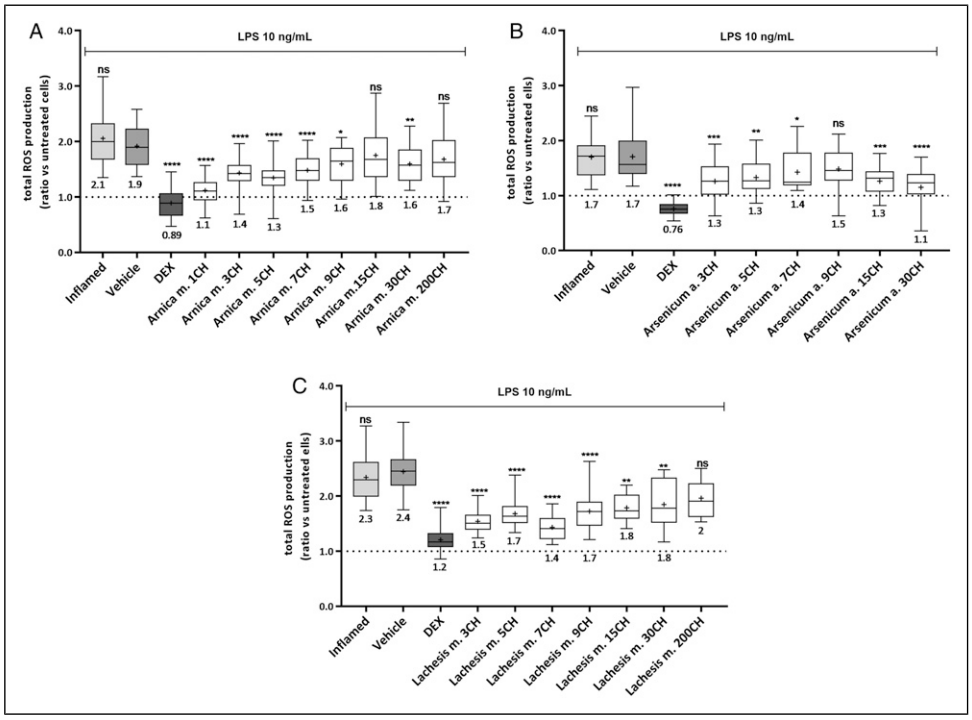

Figure 2. Effect of homeopathic dilutions of Arnica m. (A), Arsenicum a. (B), and Lachesis m. (C) on total ROS production in LPS-inflamed microglial cells. The inflamed cells were treated with homeopathic treatments for 24 h and fluorescence intensities of ROS were measured using CellROX Deep Red probe. All the values were normalized by fluorescence value of the untreated cells without LPS stimulation. The dotted line at 1 corresponds to the untreated cells. Data are represented as the mean of three independent experiments and is indicated by a cross. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 *vs* vehicle by Kruskal–Wallis test. ns: not significant. (LPS: lipopolysaccharide; ROS: reactive oxygen species; DEX: Dexamethasone).

edema induced by nyastatin where they demonstrated the efficacy of *Arnica montana* 6C when it is administrated as a pre-treatment before the inflammation.[19] The application of 20% Arnica ointment was able to reduce bruising more effectively than placebo. After a rhinoplasty surgery, a study showed that the application of local Arnica and mucopolysaccharide polysulfate cream reduced the edema and ecchymosis.[5,20] Other studies demonstrated that *Arnica montana* has an anti-inflammatory potential. The oral administration of *Arnica montana* flower methanol extract (AMME) alleviated both inflammation (TNF∝, IL-1β, IL-6, and IL-12) and nitric oxide (NO) in a collagen-induced arthritis rat model.[4] On murine macrophages, the AMME significantly reduced the protein levels of inducible NO synthase (iNOS) and the cyclooxygenase-2 (COX-2) and also decreased the production of TNFα and IL-12.[21] homeopathic dilutions of Arnica m. reduced intracellular ROS production in LPS-inflamed microglial cells. Furthermore, the action of high dilutions over Avogadro's number, corresponding to a homeopathic dilution beyond the 12C, is still present on microglial cells.

Arsenicum album. In our study, Arsenicum a. 3C, 5C, 7C, 15C, and 30C dilutions significantly decreased the oxidative stress in inflamed microglial cells. Arsenicum a. 30C dilution had the highest effect by reducing ROS production compared to the other dilutions. Arsenic can cause DNA damage and generates ROS. Arsenic exposure increases oxidative damage to lipids and proteins and decreases the levels of antioxidants. Arsenic induced cardiovascular disease, skin lesion, and immunological disorders causes inflammation and increase of oxidative stress.[22,23] Epidemiologic evidence identified a link between arsenic exposure with markers of endothelial dysfunction such as soluble ICAM-1 and soluble VCAM-1 in plasma associated with CVD.[24] On the other hand, *Arsenicum album* 30C allows greater elimination of arsenic in the urine of patients poisoned with arsenic.[25] Moreover, *Arsenicum album* 30C prevents the fever following DPT-HepB-Polio vaccination (Diphtheria-Pertusis-Tetanus).[26] *Arsenicum album* 30C administration reduced arsenic toxicity in *E. coli* by inhibiting generation of ROS.[27]

Lachesis mutus. Finally, Lachesis m. 3C, 5C, 7C, 9C, 15C, and 30C dilutions significantly reduced the oxidative stress level in microglial cells. Lachesis m. 7C had the highest effect by reducing ROS production compared to the other dilutions. When envenomation happens, the venom interacts with cells and becomes an activator of the immune cytokines such as TNFα and prostaglandins. It triggers spontaneous

hemorrhage, nausea, vomiting, diarrhea, coagulation disorders, hypotension cardiovascular shock, and renal malfunction.[28,29] *Lachesis mutus* 30C is usually prescribed for cardiovascular diseases, hypertension and therefore reduces inflammation. *Lachesis muta* 30C significantly reduces the blood pressure levels on hypertensive patients.[13]

The traditionally use of these three homeopathic medicines is to relieve inflammation. While the mechanism of action of these three homeopathic medicines is still unknown, this study demonstrates their anti-inflammatory properties through reducing ROS production.

Conclusion

Overall, these data demonstrated that the most homeopathic dilutions of *Arnica montana*, *Arsenicum album,* and *Lachesis mutus* tested have a potential effect to alleviate inflammation by reducing the oxidative stress level in LPS-inflamed microglial cells whereas the vehicle alone had no effect on oxidative stress. *Arnica montana*, *Arsenicum album,* and *Lachesis mutus* did not have the same impact on ROS production. Moreover, the effect of high dilutions of these three treatments was not dose-dependent. Our results obtained with homeopathic dilutions of *Arnica montana* support the anti-inflammatory effect already described in clinical studies.

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Author Contributions

NB participated to the conceptualization of the study. AP and JV performed the experiments. AP, JV, ST, and NB analyzed the data. ST supervised the experiments and wrote the manuscript. NB, JV, and AP critically reviewed the manuscript. All authors read and approved the final manuscript for submission.

Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this paper. All the authors are employees of Laboratoires Boiron France-Messimy, pharmaceutical company producing homeopathic medicines. This professional relationship does not imply any misconduct on the part of the authors.

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