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Acute toxicity of virgin and used engine oil enriched with copper nano particles in the earthworm

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Abstract. In spite of development of nanotechnology and creation of new opportunities for industry, new applications and products initiated by this technology may cause harmful effects on human health and environment. Unfortunately, there is no sufficient information on the harmful effects caused by application of some nano materials; the current knowledge in this field is limited solely to the nano particles but not the final products. Nano cupper particles, as one of the common materials produced in industrial scale is widely used as additives into engine oil to reduce friction and improve lubrication. However, the difference between the effects of virgin and used conventional engine oil (CEO) and the engine oil containing cupper nano particles (NEO) on the environment is not known. Earthworm, as a one of the species which could live and survive in different sorts of earth and has a certain role in protecting the soil structure and fertility, was used in this experiment. In accordance with the recommended method of OECD.1984, Filter Paper test in 24 and 48 h based on 8 concentrations in the range of $3 \times 10^{-3}$ - $24 \times 10^{-7}$ ml/cm² and Artificial Soil test in 7 and 14 days based on 7 concentrations in the range of 0.1 mg/kg – 100 g/kg were carried out to study earthworms in terms of lifetime (LC50), morphology and pathology. It was shown that the 48 h LC50 for virgin CEO, virgin NEO, used CEO(8000 km) and used NEO (8000 km) were $6 \times 10^{-3}$, $23 \times 10^{-3}$, $24 \times 10^{-3}$ and $16 \times 10^{-3}$ ml/cm², respectively. Furthermore, 14-day LC50 in artificial soil for all cases were above 100 g/kg. It is concluded that virgin CEO is more toxic than virgin NEO. Meanwhile, the CEO shows significant reduction in toxicity after consumption and the used NEO shows more toxicity in comparison to virgin product. It seems that more investigations on the effects of final products specifically after consumption is necessary because the products after consumption have the most contact with environment and subsequently human health.

1. Introduction

Conventional products are gradually replaced by nano products. Recent studies indicate the toxicity of nano materials on aquatic and rodents, however, many hidden effects of nano materials are still
unknown [1]. Therefore, a greater amount of study is required on the effects of nano products on humans and the environment [2]. Currently, there is a limited understanding about side effects of nano materials in nature and wildlife [3]. Lots of data is needed for systematic studies of nano materials’ toxicity in different species in water and soil environments [3]. Much of our information about toxicity of nano materials is on aquatic organisms, thus, there is a need for further studies on toxicity of nano-materials on land animals as well [1].

The earthworm, as a species which is able to live in different types of soil, is known as an important experimental model for monitoring the possible effects of environmental chemicals on the soil systems. Its higher sensitivity, limited mobility, and easy collection and rearing in the laboratory are among factors making it suitable for this kind of studies. Therefore, many studies have been accomplished using this species on the effects of chemicals, including heavy metals, pesticides, aromatic hydrocarbons, cadmium, nickel, MTBE, perchlorate, and lead compounds [4-12]. The available information indicates that engine oil is considered as a dangerous environmental pollutant and its use may cause destructive environmental effects around the world [13].

Because of substantial increase in the introduction and applications of nano materials and potential impacts of them on the environment as well as little information available in this field, the environmental effects of these substances are required to be identified. Copper nano particle is one of the nano metals which is commercially manufactured for application in various industries. For instance, due to its high speed, low cost and recycling properties, copper nano particle is used in the anode pole materials of the lithium batteries [14]. Moreover, as a bioactive cover, it is capable of inhibiting microorganisms such as Sacromaysis, E. coli, S. aureus and Listeria [15]. One of the other characteristics of copper nano particles is their plasticizing feature when mixed with used oils in the oil industry [16]. In general, it can be said that nano particles have restorative properties in abrasion surfaces, which can cause the friction to be reduced.

Nano cupper particles are widely used as additives into engine oil to reduce friction and improve lubrication. However, the difference between the effects of virgin and used conventional engine oil (CEO) and the engine oil containing cupper nano particles (NEO) on the environment is not known. The aim of the present study was to compare the effects of 4 conditions of the engine oil on the earthworm. The experiments were performed in accordance with the recommended protocols of OECD [17].

2. Materials and Methods

2.1. Test Animals
Earthworm specie used for testing was Eisenia foetida (Michaelsen). The worms reached to maturity level (>2 months old) and weighed 300 to 600 mg. The worms were taken from a research institute for organic vegetable wastes in the city of Tehran, Iran.

2.2. Test Substances
The engine oil used in this study was a commercial brand manufactured in Iran and enriched with copper nano particles by researchers working at Research Institute of Petroleum Industry (RRIPI), Tehran, Iran. The product was tested in 4 different conditions: virgin conventional engine oil (vCEO), used conventional engine oil (uCEO), virgin nano engine oil (vNEO) and used nano engine oil (uNEO). Copper nanoparticles had the cover in the size range of 6-20 nm and the average diameter of 12 nm. The engine oil type was SAE20W-50W with oil basis, and the density of 0.898. Used products had a performance of 8000 km.
2.3. Experimental Protocols
Acute toxicity test was done with the methods of filter paper (test) and artificial soil test according to instructions of the number 1984 (207).

2.3.1. Filter Paper Test. The filter paper had the following specifications: average grade, weight 80-85 g/m², thickness 0.2 mm, area 3×3 cm. Vials were flat-bottom glass, area 3×3 cm, height 8 cm. Two ml of the final solution (test substance in chloroform as the solvent) was inserted by pipette in each of the vials. The test substances were either 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 or 2.0 ml. Thirty animals were used for each group (every concentration of every substance). Before covering, the vials were exposed to slight flow of the air to dry. To eliminate any bias due to the effect of the physico-chemical properties of the engine oil, three extra control solutions were also used, they included olive oil, olive oil + chloroform (50:50), and water. Before the test, worms were placed for 3 h in a dry dish to defecate. Thereafter, they were placed in the vial (1 worm/vial) and 1 ml of de-ionized water was added onto the paper. Then, the vials were covered by nylon. They were left in a dark place at room temperature for 48 h. The number of dead animals as well as morphological changes was evaluated after both 24 h and 48 h.

2.3.2. Artificial Soil Test. A mixture of 10% peat moss (mulch), 20% clay and 70% sand was used as the artificial soil. The pH of the medium was set at 6.0±0.5 by adding calcium bicarbonate. De-ionised water was added to the soil to increase the moisture (350 ml of water for 1 kg of DRY soil). Test substances were added to the final soil (750 g in 1 L glass containers) at the concentrations either 0, 1, 10, 100, 1,000, 10,000 or 100,000 ppm. The worms were kept in un-treated soil for 24 h, after which they were transferred to the test medium. Ten animals were placed in every glass container and the experiment was repeated 4 times (number of animals per group = 40). The containers were left in a light place at room temperature for 14 days. Evaluation of mortality and morphological changes were performed on days 7 and 14.

2.4. Statistical Analysis
Data of each experimental group was expressed as mean ± SEM. Differences were first evaluated by one-way analysis of variance (ANOVA) and were then compared by the post-ANOVA test, Bonferroni’s t test. In addition, correlations between concentration of the engine oil and the death of the earthworm were evaluated and the regression factor was calculated (-1 > r < +1). The difference was considered to be statistically significant whenever P < 0.05.

3. Results
3.1. Mortality rates (LC50)
LC50 within 48 h in paper test for vCEO, vNEO, uCEO and uNEO were 6×10⁻³, 23×10⁻³, >24×10⁻³ and 16×10⁻³ ml/cm², respectively. In brief, the toxicity ratio was as vCEO > uNEO > vNEO > uCEO. There were correlations between concentration of the oil and response (death): vCEO (r=0.924, P=0.025), uNEO (r=0.968, P<0.001), vNEO (r=0.982, P=0.121), uNEO (r=0.954, P<0.001). Furthermore, LC50 within 14-day in artificial soil test 14-day LC50 in artificial soil for all cases were >100 g/kg. All together, in most concentrations, vCEO was found to be more toxic than all other products used (P<0.05). Figure 1 shows the details of mortality at all used concentration of 4 different types of the engine oil.
Figure 1. Charts related to mortality (deaths) in four types of oil in different concentrations:
A-Comparative effects of used & virgin NEO and CEO in paper test within 24 hours.
B-Comparative effects of used & virgin NEO and CEO in paper test within 48 hours.
C-Comparative effects of used & virgin NEO and CEO in artificial soil test within 7 days.
D-Comparative effects of used & virgin NEO and CEO in artificial soil test within 14 days.

3.2. Histopathological changes
Extensive damage to many organs were caused by engine oil in the earthworms subjected to paper test within 48 hours. Some of important observations are outlined in Table 1 and representatives of the photomicrographs are shown in Figure 2.
**Figure 2. Histopathological Effects of vCEO:** Watery degeneration, necrosis and individuation of the epidermis from its underlying tissue, along with influence the dark pigment among circular muscle cells (A). Extensive destruction of nephric tissue (B). Leucomalasia in the abdominal nerve (C). **Histopathological effects of vNEO:** Leucomalasia in the abdominal nerve (D). **Effects of uNEO:** Normal cuticle, normal circular and longitudinal muscles, and dark pigments can be seen in the digestive tract (E). **Histopathological effects of uCEO:** Normal cuticle, normal circular and longitudinal muscles, and dark pigments are seen in the digestive tract (F).

|                  | vCEO | uCEO | vNEO | uNEO |
|------------------|------|------|------|------|
| Epidermal separation | +++  | -    | ++   | +    |
| Loss of villi in the gut | +++  | -    | ++   | -    |
| Nerve leukomalacia   | +++  | -    | +    | -    |
| Tubular necrosis in the urinary tract | +++  | -    | +    | -    |
| Skeletal muscle necrosis | ++   | -    | -    | -    |
3.3. Behavioral and morphological changes

No effect was observed on the worms subjected to the artificial soil test. On the other hand, concentration-dependent decreases in response to needle and light stimulations by the survived worms were recognized during the paper test; all groups showed degrees of mucosal secretions and coiling. Except in the uCEO group, all animals showed the following signs: fragmentation, bleeding, and swelling.

![Figure 3. Morphological observations: Fragmentation (A), swelling (B), coiling (C) and bleeding (D) in the earthworms subjected to engine oil during paper test.](image)

4. Conclusion

In the present study, a full study was performed to determine the environmental impact of engine oil. In addition to the conventional form, a commercially designed product, enriched with copper nano particles was also evaluated. As the toxicity could be either enhanced or declined with the usage of these compounds, besides the virgin product, the effects of used engine oil of both forms were also investigated. Based on the protocols imposed by OECD, the earthworm was used as the experimental model.

Although not studied extensively enough, the carcinogenic action of engine oil has been understood since a half century ago [18]. The carcinogenic effect of used engine oil in mouse skin, explained 30 years later, was shown to be related to specified adducts produced following the exposure with the reactive metabolites of the product [19]. In spite of the carcinogenic evidence, used engine oil is applied on damaged human skin by unauthorized persons in Iran. It should be taken strange that it is suggested to be used in the treatment of skin disorders of farm animals [20]. A case of 64-year-old male with longstanding occupational exposure to used engine oil was reported to present with a rare intraepithelial adenocarcinoma, i.e. extramammary Paget's disease of the left scrotum and groin [21]. Evidence suggest that more caution should be taken into account in the use, destruction and recycling of all kinds of engine oil throughout the world.

Finally, the outcome of the findings in this paper can be summarized as follows: (1) Virgin engine oil can be harmful for the environment as shown in the earthworm. (2) The product is detoxified after...
conventional use in motor vehicles. Therefore, the used products are considered to be less toxic than
the virgin engine oil. (3) Engine oil becomes less toxic when enriched with copper nano particles. This
is true only in case of the virgin product and, hence, it safer for professionals working with this
product. (4) The used nano enriched engine oil is more toxic than the conventional used product for
the environment. Therefore, it has to be disposed with particular attention.

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