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Original Article

Cardiac Toxicity of Nicotine and its Synergistic Effect with Caffeine: the *Daphnia pulex* Model

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Purpose: Nicotine and caffeine are among the most frequently self-administered licit psychoactive drugs in Taiwan. This study investigated whether *Daphnia pulex* could be used to investigate the effects of nicotine and caffeine on the heart and to establish *Daphnia* as a model for investigating the role of toxins.
Methods: The toxic effects of pure nicotine and caffeine were measured and compared with the EC_{50} value of the mixed drugs using *D. pulex* acute toxicity test, chronic toxicity test, measurement of heart rate, and mixed toxicity test.
Results: The heart rate fluctuated within the first 14 days, and then it dropped dramatically on the 15th day and hit the lowest point at experiment termination. It can be concluded that heart of water fleas may respond to low concentrations of chemicals until the 15th day. With a Mixture Toxicity Index of 2.6, we found that combined caffeine and nicotine had a synergistic effect.
Conclusion: The impact of chemicals on *D. pulex* can be displayed with better clarity by incorporating heart rate analysis. The water flea can be studied as an indicator of the presence off environmental toxicants.

Keywords: Nicotine, Caffeine, Mix toxicity, *Daphnia pulex*

Introduction:

Toxicity data from tests on single pure chemicals in the laboratory provide fundamental information for the chemical risk assessment or for the formation of water quality criteria. Typically, aquatic organisms are not exposed to only a single substance, but rather simultaneously to multiple mixtures of chemicals[1]. Most studies on mixture toxicity have been separately performed with nicotine or chemical mixtures[2-4]. Although it is not well-known, many aquatic organisms are exposed to both nicotine and caffeine.

Nicotine is the main pharmacologically active ingredient in tobacco and its presence in smoke is recognized as fundamental to the initiation and subsequent persistence of smoking habits and addiction[5]. Nicotine, a naturally occurring alkaloid found in many plants, is an amine composed of pyridine and pyrrolidine rings. Its effects have been extensively investigated in animals and in a variety of cell systems. It has a wide variety of effects on multicellular organisms and single cells[2]. The

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predominant effects of nicotine in the whole intact animals or humans consist of an increase in pulse rate, blood pressure, and an increase in plasma free fatty acids, a metabolization of blood sugar, and an increase in the level of catecholamines in the blood[2,6]. At the cellular level, the effects include increased expression of heat shock proteins, induction of sister chromatide exchanged and chromosome aberration, inhibition of cell proliferation, and suppression of apoptosis[2-3,7].

Another common toxin consumed by human is caffeine, mostly derived from the consumption of coffee or tea, but also present in a large number of foods, beverages and over-the-counter drugs. It acts as a central nervous system stimulant and also has negative withdrawal effects[8]. In large doses, it can be profoundly toxic, resulting in arrhythmia, tachycardia, vomiting, convulsions, coma and death[9]. Caffeine has been purified and was found to be responsible for the stimulant action of coffee. Because of its stimulatory nature, it was used as a cardiotonic till the end of 19th century. In modern medicine, caffeine is used as an adjuvant to the analgesic actions of aspirin and paracetamol[8]. Low levels of human pharmaceuticals have been detected in many countries in sewage treatment plant effluents, surface waters, seawaters, groundwater and some drinking waters[10]. Both of the nicotine metabolite cotinine and the stimulant caffeine are generally present in sewage treatment plant and surface waters[11].

The cladocerans *Daphnia pulex* have been established as a useful model in ecotoxicology and the investigation of the ecological impact of toxic substances in freshwater[12]. These cladocerans are sensitive to a wide range of aquatic contaminants, and because they have a relatively short life cycle and require little space, they are adaptable to laboratory conditions[13]. Their transparency allow us special insight into animal physiology, as it allows the researcher to apply optical methods to visualize physiological functions and to measure several different parameters simultaneously[14] while at the same time engage in non-invasive experimentation.

Because water bodies are contaminated with multiple toxicants[15], the interactivity of chemicals producing a toxic response needs to be considered when assessing the impact of toxicants on the aquatic environment. While few studies had investigated the effect of nicotine and caffeine separately on the heartbeat the aquatic organisms, no ecotoxicologic study has investigated the combined toxicity of nicotine and caffeine in *Daphnia* toxicity. The study was performed to show whether *D. pulex* can be used to investigate the effects of nicotine on the heart and its toxic effects when combined with caffeine.

Materials and methods:

Chemicals

Nicotine [3-(1-methyl-2-pyrrolidinyl) pyridine, $C_{10}H_{14}N_2$], and caffeine [(3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6 dione), $C_8H_{10}N_4O_2$] were purchased from Merck company (Germany).

Daphnia cultures

Daphnia pulex (*D. pulex*) have been maintained parthenogenetically in Chung Shan Medical University, Taiwan since 2001. They are kept in 10-liter tanks on the window ledge of a laboratory at approximately 20°C. The tanks generate enough green alga (*Chlorella vulgaris*) to sustain a colony of several hundred for at least 6 months. The walls of the tanks are scraped clean every 2-3 weeks and topped up alternatively with dechlorinated and conditioned tap water to replenish water lost by evaporation. The water in the takes are aerated by filtered air. The *Daphnia* were observed in a specially constructed chamber (maintained in most experiments at 20±1°C) using a binocular dissecting microscope (magnification 20-60×). The *D. pulex* were approximately 1-2 mm in length and their hearts 100-200 µm long and 50 µm across in size. Their heart is situated above the brooding chamber, making it appear at first glance to swim upside down while feeder moves food into its mouth facing upwards. In order count heart beats, the animal was viewed with highest magnification (60×). Each *D. pulex* was maintained within a 50-µl droplet throughout the experiment.

Measurement of heart rate

A wide range of artificial media have been developed to study *Daphnia* spp.[12,20]. Here *D. pulex* were incubated in 50-µl drops of either dechlorinated and conditioned tap water or a simulated high hardness medium containing the agent being studied. *D. pulex* thrived in both media for several hours with no significant change in heart rate. Between five and seven droplets were used per Petri dish to perform each experiment. The *D. pulex* were free to swim

around in each droplet. However, it was necessary to cool the *D. pulex* since their heart rates increase so much at room temperature that it would be impossible to obtain accurate measurements at that temperature. The heart rate was counted manually at 15-s intervals and the mean was converted to beats per second. These were consistently within 5% of each other. The mean was then converted to percentage of time for each condition. The change in heart beat calculated as percent change from baseline of initial control group.

Acute toxicity tests

All procedures were followed as specified by USEPA[16]. The immobilization test procedures were utilized to evaluate the acute toxicity of nicotine and caffeine. *D. pulex* was exposed to nicotine at nominal concentrations ranging from 0 (control) to 0.6, 0.9, 1.3, 2.0, 3.0 mg/L, and they were was exposed to caffeine at nominal concentrations ranging from 0 (control) to 31, 62, 125, 500 mg/L. Tests were performed with four replicates each containing five neonates (<24 h old) in 50 ml of medium in 100 ml glass beakers. All experiments were conducted in an environmental chamber under a constant temperature of 20±2°C and a short photoperiod(8-h light:16-h dark). Temperature, dissolved oxygen, pH and Daphnids' heart beat were monitored at 0, 24, and 48h. Immobility(synonymous with death) was observed daily.

For acute toxicity tests, simulated high hardness medium(0.77 mM $CaCl_2 \cdot 2H_2O$, 0.46 mM $MgSO_4$, 0.01 mM K_2HPO_4, 0.20 mM $NaNO_3$, 1.50 mM $NaHCO_3$, 0.11 mM $Na_2SiO_3 \cdot 9H_2O$, 0.39 mM H_3BO_3, 0.08 mM KCl, pH 7.0) was prepared from deionized and distilled water. After aeration, the pH was adjus ted to7.4-7.5 with 1N HCl before use. Immobility was used as the endpoint indicating acute toxicity. Daphnids showing no movement within 15s after gentle stirring were defined as immobile. The 48-h EC_{50}(median effectiveness concentration) values and 95% confidence limits were calculated by Probit analysis[17], based on nominal concentrations.

Chronic toxicity test

Daphnids(<24h) were exposed to nicotine at nominal concentrations ranging from 0.08, 0.12, 0.18, 0.27, to 0.40 mg/L, calculated based on the results obtained from preliminary acute immobilization test. Each treatment group and the control group consisted of two 250 ml beakers containing 100 ml of medium and ten daphnids. All experiments were conducted in an environmental chamber under constant temperature(20±2°C) and a short photoperiod(8-h light:16-h dark). Solutions in beakers were renewed three times per week, at which time we added 1.5×10^6 cells of green algae(*Chlorella vulgaris*) to each beaker the first week and 3.0×10^6cells thereafter.

Daphnids were checked daily until the day eggs were first observed in the brood chamber, indicating attainment of maturity. The number of molts was also monitored daily until first brood. Survival, ecdysis, and offspring production were assessed at the time of media change. During the experiments, temperature, dissolved oxygen, and pH were monitored weekly. Daphnids' heart beat were monitored daily. Endpoints included 21 day adult survival, with total number of live and dead neonates counted in 21 days. During each renewal period, live and dead young were counted and discarded[18].

Mixed toxicity test

For a more convenient interpretation of toxicity data, the results obtained as the lowest-observed-effect concentration(LOEC) values were converted into a mixture toxicity index(MTI) to compare quantitatively the results of the mixture toxicity tests. MTI is calculated as follows: $MTI = 1 - \log M / \log n$, where $M = \Sigma TUi$. This index was proposed based on the assumption that the chemicals in the mixture act with simple, similar, and independent actions. MTI was calculated using a toxic unit(TU), defined by Sprague and Ramsay (1965) as $TU = [1/EC_{50}]$, and considering the number of compounds in the mixture(*n*). Effects of the mixture can be evaluated by this index in the toxicity scale proposed by Könemann[19].

All procedures followed those specified by USEPA[16]. Immobilization test procedures were utilized to evaluate the mix toxicity of nicotine and caffeine to *D. pulex*, following the same procedures of as described for the acute toxicity test above. The concentration of the test was mixed with the LOEC of nicotine(0.6 mg/L) 25 ml and caffeine(31 mg/L) 25 ml. Tests were performed with four replicates each containing five neonates(<24 h old) in 50 ml of medium in 100 ml glass beakers. All experiments were conducted in an environmental chamber under a constant temperature(20±2°C) and with short photoperiod(8-h light:16-h dark). Temperature, dissolved oxygen, and pH were monitored at 0, 24, and 48h. Immobility(synonymous with death) was observed daily.

Statistical analysis

The EC_{50} values and 95% confidence limits were calculated by probit analysis using SAS statistical software, version 9.1.3 (SAS Institute, Inc., Cary, NC).

Results:

Susceptibility of *Daphnia pulex's* heart to the chemicals

The heart of *Daphnia pulex* can serve as an important indicator of organism response to environmental changes. As seen in Fig.1, during the first 30 minutes of the nicotine serial concentrations experiment, there were signs of reaction in the heart. Heart rates also tended to increase. During the nicotine concentration series test, heart rates increased from 0.7-8.6% at concentrations of 0.6, 0.9, 1.3, 2.0, and 3.0 mg/L, respectively, compared with the control group(0 mg/L). However, from 30 to 60 minutes, heart rates of the experimental group began to drop, while those of the control group go on constantly. The heart rates of the experimental group were down by 1.3-2.2% compared to those of the control group at the 30 to 60 minute of the experiment. Between the 30th to 90th minute, heart rates of the experimental group began to rise in contrast to the decrease in heart rates of the control group. By the time the experiment came to an end after 90 minutes, heart rates of the water fleas with a reagent concentration of 2 mg/L had dropped by 0.7%, while those for the test group with a reagent concentration of 0.9 mg/L appeared unchanged. Those with remaining concentrations were found to have slight increases, ranging from 0.3 to 1.9%.

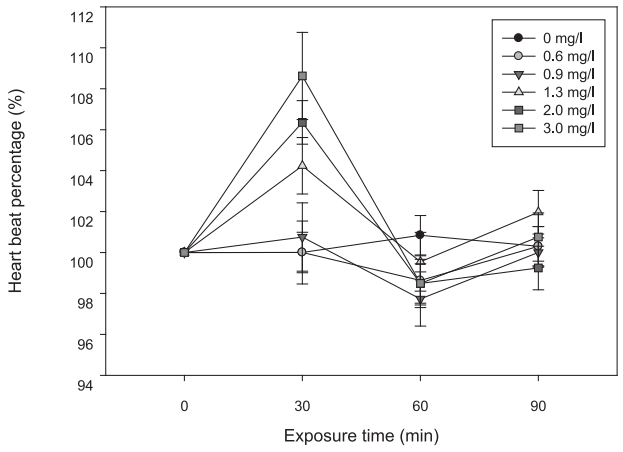


Fig.1. The relationship of nicotine solution exposure and heart beat percentage of *D. pulex*.

Influence of nicotine on the heart rate of *Daphnia pulex*

Acute toxicity test

Toxicity values of nicotine and caffeine on *D. pulex* neonates are summarized in Table 1. The 48-h EC_{50} and 48-h LOEC values of nicotine were 0.6 and 2.11 mg/L, respectively. Those of caffeine were 31 and 178.95 mg/L. By the time the test was terminated at 48 hours, heart rates had increased by 6%, 8%, 10%, 12% and 17% for the solutions with nicotine concentrations of 0.6, 0.9, 1.3, 2.0 and 3.0 mg/L respectively, significantly different from the control group(0 mg/L)(p<0.001)(Fig. 2a), this is a negative correlation. As can be seen in Fig. 2b, a decrease in heart rate was negatively correlated toxin concentration.

Table 1. Toxicity values of nicotine and caffeine on *D. pulex* neonates after 48-h exposed

Reagent	LOEC	EC_{50}
Nicotine	0.6	2.11
Caffeine	31	178.95

LOEC: lowest observed effect concentration, mg/L
EC_{50}: Median effectiveness concentration, mg/L

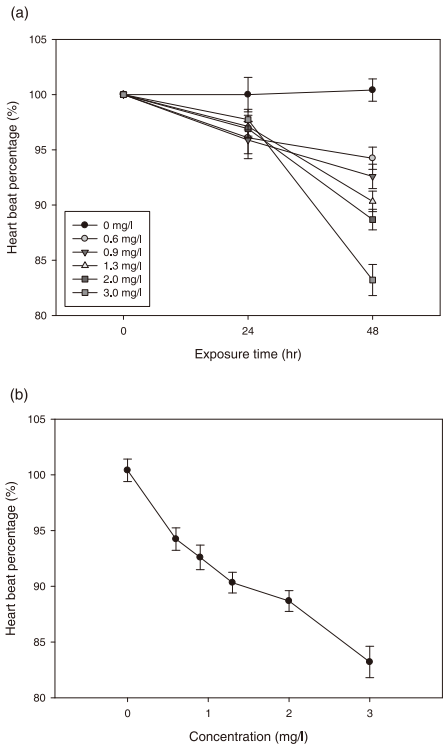


Fig. 2. The influences of nicotine on the heart beat of *D. pulex*. (a) Showed a dramatic difference in comparison with the control group. (b) The influence of the nicotine concentrations on the heart beats of *D. pulex*, which indicated the existence of a positive correlation.

Chronic toxicity test

The chronic toxicity test utilizes methods previously described by Organization for Economic Cooperation and Development(OECD) in 1998. Standard deviations were left out to avoid confusion interpreting the graph(Fig. 3). The heart rates of the water fleas had a tendency to increase on the 2nd, 5th, 9th, 11th and 13th day, peaking on the 9th day. The heart rates of the experimental group increased from 0.6 to 8.8% in solutions with nicotine concentrations of 0.08, 0.12, 0.18, 0.27, 0.40 mg/l, respectively, compared to the control group(0 mg/L). On the 14th day of the test, their heart rates began to drop. They had dropped to minimal levels by 21st day, when the test was terminated. The heart rates of the water fleas of the experimental group dropped by 7-9% which comparison to those of the control group.

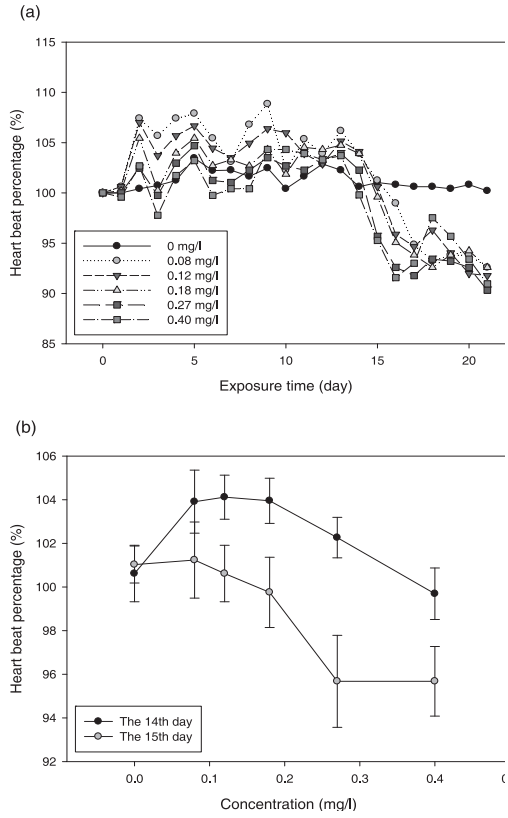


Fig. 3. The trend of long term exposure that *D. pulex* exposure in nicotine solution. (a) Showed the trend of heart beat percentage with time exposure. (b) Showed the trend of heart beat percentage with serial concentrations, the significance differentiates from 14th and 15th days (p<0.001).

Mix toxicity test

$TU = 1/EC_{50}$, $i = 2 \rightarrow TU = 1/$nicotine 48h-LOEC $+ 1/$caffeine 48h-LOEC

$M = \Sigma TUi \rightarrow M = 1/0.6 + 1/31 = 1.699$

$MTI = 1 - \log M/\log n \rightarrow 1 - \log 1.699/\log 2 = (1 - 0.230)/0.301 = 2.6$

The detection of toxicity of chemical mixtures is very important in ecotoxicology since a mixture of toxic substances may have a more adverse effect on the environment. For convenience of calculation, we frequently used Mixture Toxicity Index(MTI) to measure mixture toxicity of chemicals. The MTI makes it easier to clarify relationships among chemical reagents(i.e., synergism or antagonism). The research with a MTI of 2.6 indicates that caffeine can add to the toxicity of nicotine(Table 2). Furthermore, we conducted mixture toxicity experiments with reagents of low concentrations(nicotine, 0.6 mg/L and caffeine 31 mg/L)(Fig. 4). The toxicity of either caffeine or nicotine in the mixture was 15% higher than that of a single chemical. Low concentrations of nicotine and caffeine were lethal to 50% or over in the acute toxicity test. The LC_{50} value of the mixtures equaled that of the single reagents. Results from the mix toxicity test showed that *Daphnia* heart beat test was more susceptible to EC_{50} value when used in the MTI calculation.

Table 2. Mixture toxicity scale after Könemann.

MTI	Classification of toxicity mixtures for possible type of joint action
MTI < 0	Antagonism
MTI = 0	No addition (independent action, r = +1)a
0 < MTI < 1	Partial addition
MTI = 1	Concentration addition (simple similar action)
MTI > 1	Supra-addition (potentiation of the toxic action(s) of one or more of the compounds of mixtures)

a Positive correlation between susceptibilities of the individual organism to the single toxicants. (Hernando et al., 2003)

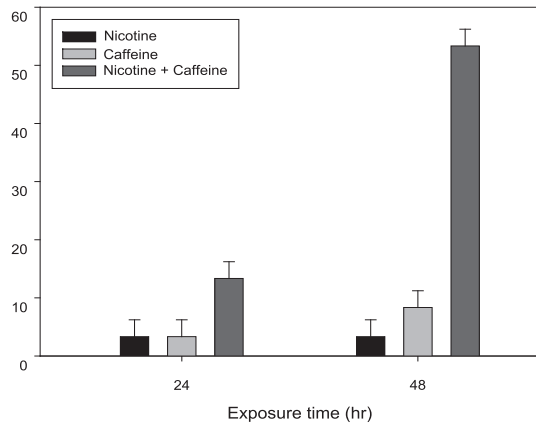


Fig. 4. Heart beats response of *D. pulex* with nicotine (2.11 mg/L), caffeine (178.95 mg/L), and mixture (nicotine 0.6 mg/L and caffeine 31 mg/L), compared with time exposure.

Discussion:

Both of the nicotine metabolite cotinine and the stimulant caffeine are generally present in sewage treatment plant and surface waters[11]. The 48h-EC_{50} values of nicotine and caffeine in acute toxicity test were 2.11 and 178.95 mg/L respectively. Exposure pathways, target organs and toxicological mechanisms vary depending on the exposed mammal, crustacean and fish, though water fleas and fish are used in preliminary detection studies before mammals were used. Water fleas are more susceptible than fish to chemicals, as it takes 50% less time for them to complete the tests(48 hours vs. 96 hours)[21]. More endpoints are needed for the development of fast toxicity detection methods. They could include, for example, photokinetic movement, enzyme activity and protein action in chemical or biochemical reaction. The heart of the transparent *D. pulex* is susceptible to the surrounding environment. Their heat rates have been observed to rise once exposed to added substances, as do the heart rates and rhythms of mammals exposed to similar substances[4,12]. Susceptibility of the *D. pulex's* heart in our study is shown in Fig. 3. Reactions were first observed 30 minutes after the beginning of the experiment. The heart rates were found to be minimal at the 60th minute, a finding that corresponds to the studies of Campbell et al. in 2004. Despite the dissimilarities between water fleas and mammals, their hearts may exhibit the same reactions to some chemical reagents.

Villegas-Navarro et al. (2003) pointed out that Ouabain, metaproterenol and metoprolol influenced the hearts of water fleas and human beings in a similar way. Crozier et al. (1999), like the present study, reported that nicotine decreased the heart rate of water fleas[4]. Although chronic toxicity is often ignored in previous studies, we proceeded to follow toxicity and found that the heart of *D. pulex* also responded to low concentrations of chemicals(0.08, 0.12, 0.18, 0.27, 0.40 mg/L respectively). We found that although the heart rate fluctuated within the first 14 days, it dropped dramatically on the 15th day reaching minimal levels by the termination of the experiment(Fig. 2), suggesting that the threshold tolerance of *D. pulex's* heart to low-concentration reagents may be reached on the 15th day.

The evaluation of the impact of environmental toxicants on the environment, however, should never be limited to single chemicals, for contamination of environmental water with toxicants varies[15,22]. Based on a mixture toxicity index of 2.6, previous studies have found that combined nicotine and caffeine have a synergistic effect, and evaluation of synergism among a variety of chemicals would be helpful in determining pollution tolerance limits in aquatic ecosystems[15,22]. For the assessment of the contamination in aquatic ecosystems, many types of bioassays have been used. The test organisms incorporated in these bioassays include species of different groups of microorganisms, plants, invertebrates and fish. Zooplankton (in particular the genus *Daphnia*) is frequently used in ecotoxicological tests because they have been found to be most sensitive to toxic chemicals and because they occupy a central position in the lentic food chain[23]. They play an significant role in static water food chains[15], they are easy to rear, and they have a short life cycle, differentiated ages and high transparency, so measurement requires only simple operations which can be performed at lower costs[12]. We conclude they water fleas can play important role in environmental toxicant detection. Incorporating heart rate in the analysis will better clarify the impact of chemicals on this organism.

Conclusion:

The heart rate fluctuated within the first 14 days, and then it dropped dramatically on the 15th day and hit the lowest point at experiment termination. It can be concluded that heart of water fleas may respond to low concentrations of chemicals until the 15th day. With a Mixture Toxicity Index of 2.6, we found that combined caffeine and nicotine had a synergistic effect.

Our results support previous reports suggesting that the responses of *Daphnia pulex* heart to pharmacological substances can be used as endpoint indicators. This notion has lead to the wide use of *Daphnia* as a tool of chronic toxicity exposure monitoring. Their use saves 70% of detection time of traditional chronic toxicity tests. Studies should not test single toxicants alone but focus on their combined toxicities when mixed with other toxicants, similar to how they are found in the environment. For several mixtures, toxicity could be detected at concentrations lower than the concentration of EC_{50} values commonly detected in the environment.

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尼古丁及咖啡因的混合對心臟的毒性影響：圓水蚤(*Daphnia pulex*)模式

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目的：尼古丁和咖啡因在台灣是屬於可以自行管理的神經興奮劑。本研究的主要目的是以圓水蚤的心跳變化來探討尼古丁和咖啡因的毒性影響，並闡述以水蚤的模式研究環境的有毒物質 。

方法：以圓水蚤微生物試驗體材，及採用純尼古丁和咖啡因藥劑進行毒性試驗，比較急毒性、慢毒性、混合毒性等試驗的半數致死數值，及測量其心跳數率的改變。

結果：本研究發現在低濃度的情況下圓水蚤的心跳有明顯的反應；在第14天以前心跳呈現不規律，到第15天其心跳則呈現急速下降直到試驗結束，因此推斷圓水蚤對低濃度的尼古丁和咖啡因的耐受閾值在第15天。有毒物質對環境的影響並非單一毒物；應該是複雜的混合毒物，以混合毒物指標2.6的尼古丁和咖啡因加成作用發現對圓水蚤的影響最大，證實圓水蚤心跳速率的分析可以做為環境有毒物質的參考。

結論：本研究結果支持先前的研究報告；圓水蚤的心跳速率可以作為藥理學探討的指標。以水蚤作為廣泛的慢毒性暴露追蹤，可比傳統的慢毒性試驗節省70%的時間。

關鍵詞：尼古丁、 咖啡因、 混合毒性、圓水蚤

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