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Original Article

Effects of sublethal exposure to new pesticides lufenuron and flonicamid on common carp, *Cyprinus carpio*, hydromineral balance to further saltwater exposure

Melika Ghelichpour, Ali Taheri Mirghaed *

Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Abstract: The effects of 21 days exposure to lufenuron and flonicamid were investigated on common carp responses to saltwater exposure. Fish were assigned in three groups: control (21 days in freshwater), lufenuron (exposed for 21 days) and flonicamid (exposed for 21 days). After 21 days, all fish were subjected to 15 g/l saltwater for further 4 days. Plasma glucose, cortisol, sodium, potassium, chloride, calcium and phosphorus levels were monitored 0, 24, 72 and 120 h after saltwater exposure. Type of pesticides and time of salt water exposure had interaction on plasma glucose and cortisol levels. These two stress indicators were increased significantly in all groups after saltwater exposure. The cortisol elevation continued until 72 h after salinity challenge and then decreased significantly after 120 h salinity exposure in all treatments. Plasma glucose level showed fluctuation during saltwater exposure. It was increased after 24 h saltwater exposure, then decreased after 72 h and again it was increased after 120 h salinity challenge. Increased plasma sodium showed significant elevation along with elongation of saltwater exposure. Pesticide exposure significantly affected plasma chloride levels as the flonicamid group had significantly lower chloride compared to the control and lufenuron groups. Plasma chloride showed significant elevation along with elongation of saltwater exposure. Pesticide and time of salinity challenge had interaction on plasma calcium levels as 24 h after salinity challenge calcium level of pesticide groups increased significantly. Along with elongation of saltwater exposure, calcium level of pesticides treatments decreased but it higher than the pre salinity challenge. Plasma phosphorus level increased 24 h after saltwater exposure and decreased along with elongation of saltwater exposure. In conclusion, lufenuron and flonicamid induce stress and alter gill function and blood ionic homeostasis during saltwater exposure.

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Introduction

Now a days, input and distribution of pollutants to ecosystems and their effects are of the main environmental concerns. Industrial developments and population growth led to chemical pollutant accumulation in aquatic ecosystems (Saghali et al., 2014). The pollutants behaviors may be assessed at three levels: water column, sediments and biomass of aquatic organisms (Saghali et al., 2014). One of the serious threats for human is pollutant entry to waters, leading to accumulation in aquatic organisms' body and moving towards higher levels of food chain (Shaw and Handy, 2011). Agricultural pesticides are considered as one of the largest group of environmental pollutant, which are extensively studied in aquatic toxicology (Wang et al., 2015).

Pesticide exposure can affect fish responses to further stress. HPI is involved in this case and studies have shown pesticide exposure significantly affects cortisol responses (Bonga and Lock, 1991). The effects of the pesticides on fishes are of important environmental concerns. Flonicamid (IKI220; N-cyanomethyl-4-trifluoromethylnicotinamide), a pyridinecarboxamide compound, is a novel systemic insecticide with selective activity against hemipterous pests, such as aphids and whiteflies, and thysanopterous pests (Staetz et al., 2006). Lufenuron, a benzoylurea pesticide, inhibits the production of chitin in insects. Without chitin, a larval flea will never develop a hard outer shell (exoskeleton). Lufenuron is

*Correspondence: Ali Taheri Mirghaed
E-mail: mirghaed@ut.ac.ir
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also sold as an agricultural pesticide for use against lepidopterans, eriophid mites, and western flower thrips.

Common carp, *Cyprinus carpio*, is an economically important species rearing in many parts of Iran (Hosseini and Hoseini, 2012). It is a popular fish in Iran and its population in the Caspian Sea is supported by stock rehabilitation activities of Iranian Fisheries Organization, because of declining its natural population. This species may face saltwater stress during stock rehabilitation. There are no data about lufenuron and flonicamid biochemical effects in common carp when exposed to saltwater. Thus, the aim of the present study was to study the effects of 21 days exposure to lufenuron and flonicamid on biochemical responses to saltwater exposure in common carp.

Materials and Methods

Fish and pesticides preparation: A total number of 600 juvenile common carp with an average weight of 48±4.25 g were stocked in 2000 L fiberglass tank and acclimatized under laboratory condition for two weeks (in research station of Gharahsoo, Bandar Turkeman, Iran). During adaptation period, water was aerated persistently and it was exchanged about 75 percent daily. The fish were fed (1.5% of body weight) twice a day with commercial carp feed (Mazandaran Animal and Aquatic Feed Co., Sari, Mazandaran, Iran). Physico-chemical parameters of water monitored daily during the experiment by Hach HQ40d portable apparatus (Loveland, Colorado, USA); Temperature (27.5±1.25ºC), dissolved oxygen (7.1±0.84 mg L^{-1}), salinity (2.63±0.15 g L^{-1}), and pH (8.5±0.25). Total hardness 300±17.5 mg L^{-1} (as $CaCO_3$), alkalinity 350±20.3 mg L^{-1} (as $CaCO_3$), and calcium 110±11.7 mg L^{-1} were measured by a Portable photometer (Wagtech 7100, Berkshire, UK).

LC50-96-h determination: Lethal concentration of lufenuron and flonicamid pesticides were determined using OECD (1992) procedure. According to pre-treatment test, 5 different concentrations of each pesticide were considered. A total of 300 common carp were randomly distributed in 30 fiberglass tanks (15 tanks for each pesticide; 3 tanks per concentration and 5 concentrations per pesticide) with a volume of 160 L. The fish were exposed to concentrations of 0 (control), 5, 10, 15, 20 ppm lufenuron and 0 (control), 30, 40, 50 and 60 ppm flonicamid. Water exchange was 75 percent daily and required amounts of pesticides were added to each tank to set concentrations. The other physico-chemical parameters of water and fish feeding were same as the acclimation period. During the experiment, the number of dead fish were recorded after 96 h exposure to lufenuron and flonicamid.

Chronic experiment and salinity challenge: In this part of the experiment, 12 fiberglass tanks were assigned for 3 different treatments (4 tanks per treatment); control (without any pesticides), 10% of LC50-96-h lufenuron (0.11 mg L^{-1}), 10% of LC50-96-h flonicamid (4.3 mg L^{-1}). Each tanks stocked with 25 fish which exposed to sub-lethal concentrations of the pesticides for 21 days. At the end of the pesticides exposure period, feeding was ceased and all groups were subjected to saltwater (addition of 12 g NaCl to one litter of water) over 120 h. This salinity was chosen according to a preliminary experiment to determine tolerable salinity for the experimental fish.

Biochemical analysis: Blood samples of all groups were taken 0 (just before the salinity appending), 24, 72, and 120 h after the salinity challenge from caudal vein (six samples at each point). Two fish per tank were sampled randomly with a dip net and immediately anesthetized with 100 mg L^{-1} eugenol (Hoseini et al., 2015) within 1 min. Blood samples were taken using heparinized syringes and plasma was separated after 10 min centrifugation (1000×g), and maintained at -80ºC until analysis. All the experiments were conducted under a protocol accepted by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

Plasma levels of chloride, calcium, phosphorus and glucose were determined photometrically using Pars Azmun kits (Hoseini et al., 2016) and Zist Shimi kits (Tehran, Iran) (Hoseini and Tarkhani, 2013). Plasma samples were assayed for sodium and potassium using a flame photometer (SEAC, Florence, Italy) (Hoseini

and Tarkhani, 2013). Plasma cortisol was determined by ELISA method based on competition principle using a commercial kit (IBL, Gesellschaft für Immunchemieund Immunbiologie, Hamburg, Germany).

Statistical analysis: The mortality of the fish was subjected to Probit analysis to calculate the LC_{50}. The plasma data were subjected to two-way ANOVA and Duncan tests. Mean values were considered significantly different when $P<0.05$. Data are presented as mean ± standard error. All analysis were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, US).

Results

In the present study, LC50-96h lufenuron and flonicamid were determined 1.1 mg/L and 43 mg/L, respectively. According to the results, the pesticide exposure significantly affected plasma glucose levels as the control group had significantly lower glucose compared to the pesticide groups. Also, time of saltwater exposure significantly affected plasma glucose levels. Plasma glucose level of the control group significantly increased 24 h after saltwater exposure. Plasma glucose level of the control group decreased and again increased significantly in 72 and 120 h after salinity challenge, respectively. In the lufenuron treatment, the highest level of plasma glucose was after 24 h exposure to the saltwater and it was decreased over time. Salt water exposure caused a significant elevation in flonicamid glucose level 24 h after salinity challenge and decreased along with elongation of saltwater exposure up to 120 h (Fig. 1).

The difference between plasma cortisol levels of pre challenge (0 h) treatments was similar to the plasma glucose levels. Bar chart shows plasma cortisol of the control group and pesticide treatments significantly increased in exposure to 24 h salt water compared to the pre challenge and remained high within 72 h salinity challenge. Plasma cortisol level of the control group and pesticide treatments decreased significantly after 120 h saltwater exposure in such a way there were no differences in plasma cortisol of each treatment compared to the pre challenge. Also,

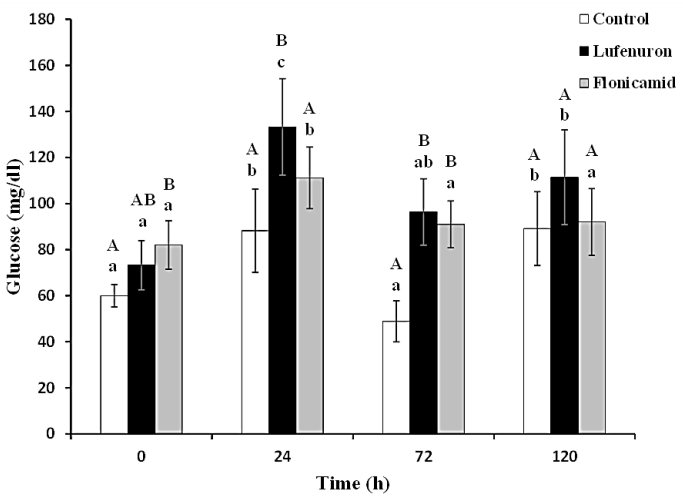

Figure 1. Effects of pesticide exposure on plasma glucose response to a 120-h saltwater exposure in common carp. Uppercase letters indicate significant difference among the treatments at each time. Lowercase letters indicate significant difference among each treatment in all times (n=6, $P<0.05$).

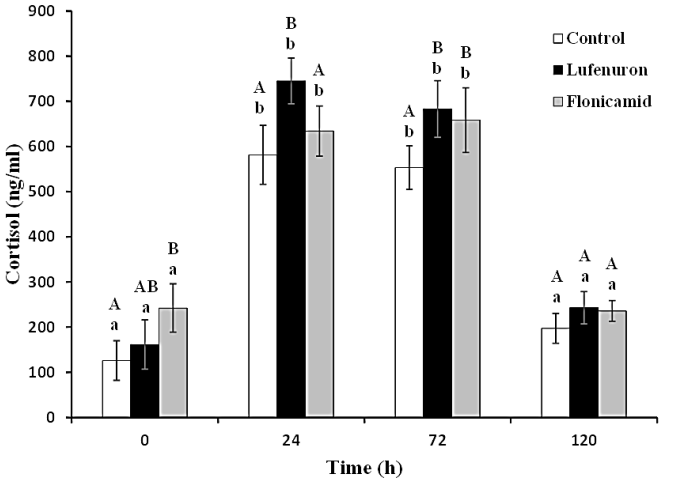

Figure 2. Effects of pesticide exposure on plasma cortisol response to a 120-h saltwater exposure in common carp. Uppercase letters indicate significant differences among the treatments at each time. Lowercase letters indicate significant differences among each treatment in all times (n=6, $P<0.05$).

120 h after exposure to the salt water, there were no differences between plasma cortisol of the control group and pesticide treatments (Fig. 2).

Figure 3 shows pesticides had no significant effects on plasma sodium, but time of saltwater exposure significantly affected plasma sodium levels. Plasma sodium showed significant elevation along with elongation of saltwater exposure. Plasma potassium level had a fluctuation along with elongation of saltwater exposure. Flonicamid affected plasma

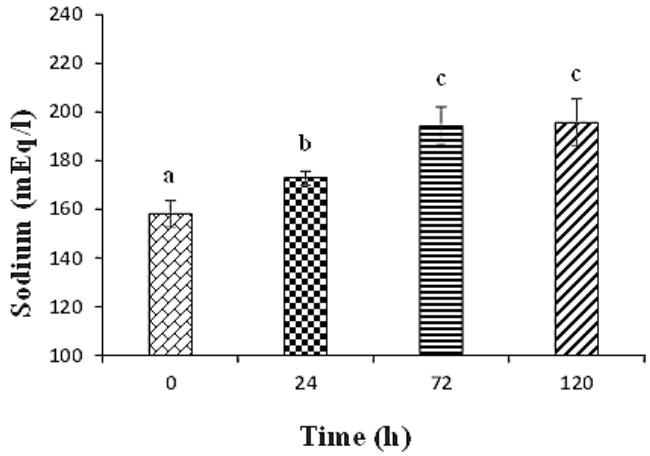

Figure 3. Effects of pesticide exposure on plasma sodium response to a 120-h saltwater exposure in common carp. Different letters show significant differences among the time exposure (n=6, P<0.05).

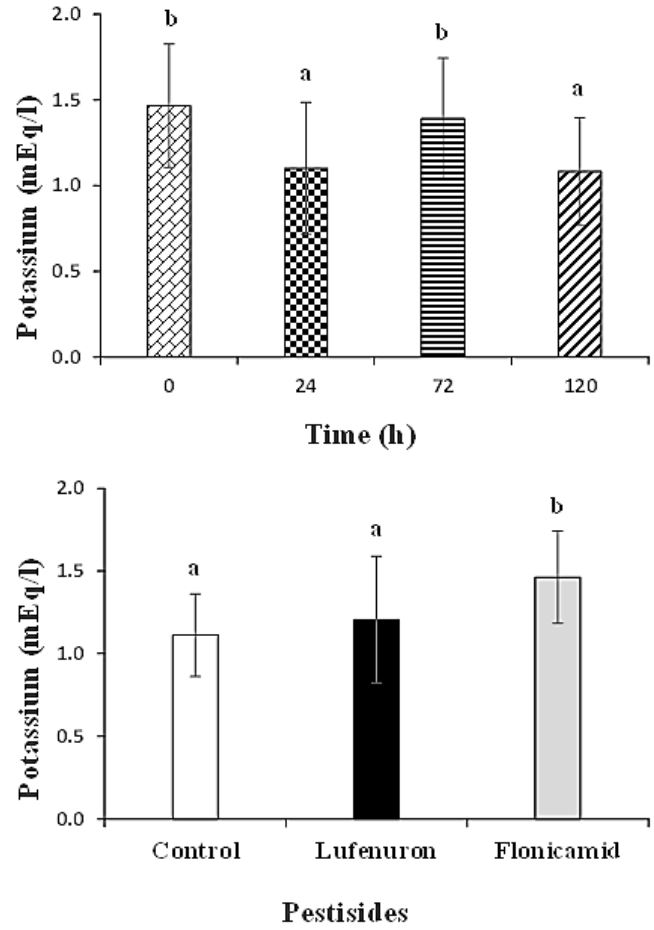

Figure 4. Effects of pesticide exposure on plasma potassium response to a 120-h saltwater exposure in common carp. Different letters show significant differences among the time exposure and treatments (n=6, P<0.05).

potassium, so that plasma potassium level of flonicamid treatment was significantly higher than the lufenuron and control groups (Fig. 4).

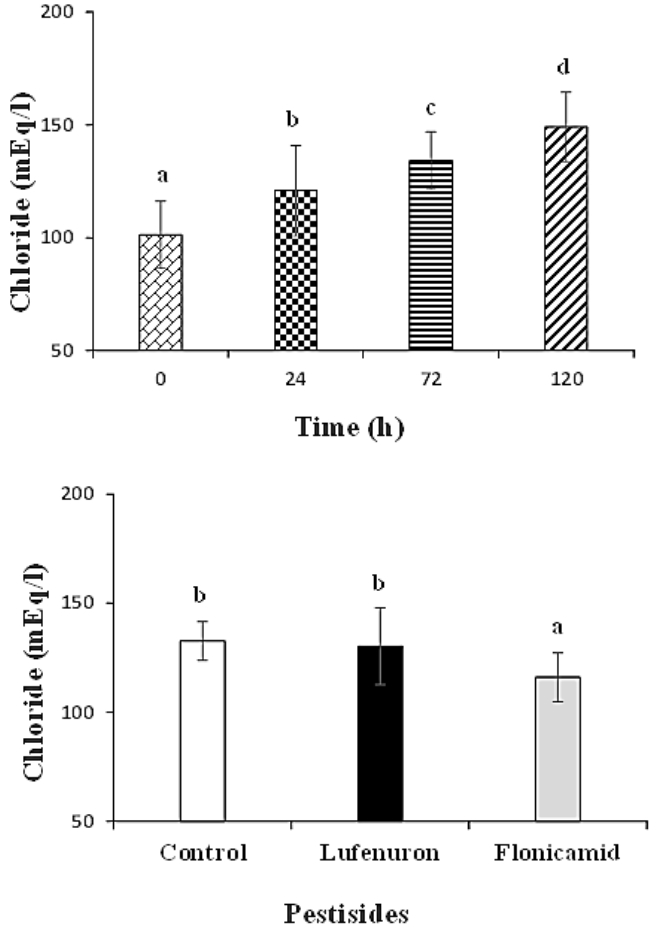

Figure 5. Effects of pesticide exposure on plasma chloride response to a 120-h saltwater exposure in common carp. Different letters show significant differences among the time exposure and treatments (n=6, P<0.05).

Pesticide exposure significantly affected plasma chloride levels as the flonicamid group had significantly lower chloride compared to the control and lufenuron groups. Time of saltwater exposure significantly affected plasma chloride levels. Plasma chloride showed significant elevation along with elongation of saltwater exposure (Fig. 5).

The results show that the kind of pesticides and the time of exposure to the salinity challenge had interaction on plasma calcium levels. Plasma calcium level of fish exposed to lufenuron and flonicamid increased significantly after 24 h exposure to the salt water. More elevation of plasma calcium level were observed in flonicamid fish exposure after 72 h salinity challenge. However, after 120 h exposure to the salt water calcium level were decreased in both pesticides groups (Fig. 6).

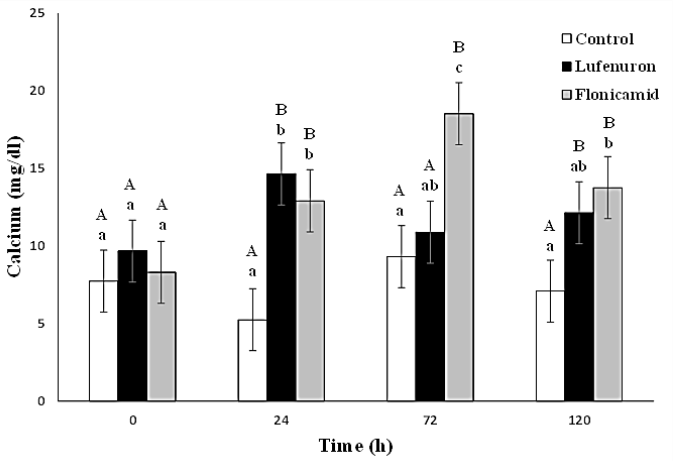

Figure 6. Effects of pesticide exposure on plasma calcium response to a 120-h saltwater exposure in common carp. Uppercase letters indicate significant differences among the treatments at each time. Lowercase letters indicate significant differences among each treatment in all times (n=6, P<0.05).

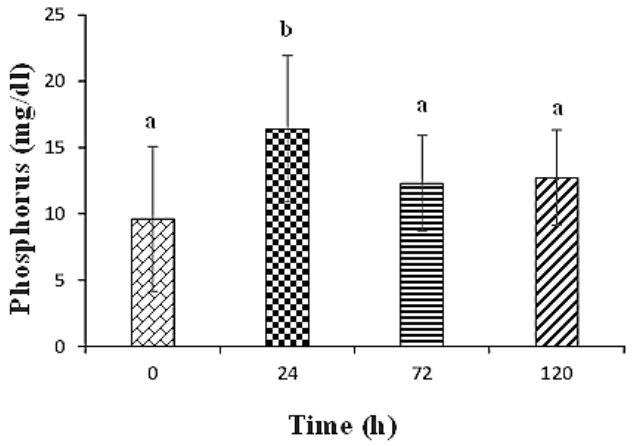

Figure 7. Effects of pesticide exposure on plasma phosphorus response to a 120-h saltwater exposure in common carp. Different letters show significant differences among the time exposure (n=6, P<0.05).

Duration of exposure to salinity affected plasma phosphorus level, so that it is significantly increased after 24 h exposure to saltwater. Along with elongation saltwater exposure (after 72 and 120 h) plasma phosphorus decreased significantly with no difference compared to the control group (Fig. 7).

Discussions

Plasma glucose and cortisol are indicator of stress in fish (Bonga, 1997; Barton, 2002). Stressors cause temporary increase in plasma cortisol levels to provide demanded energy to deal with stress during stressful conditions (Bonga, 1997, 2002; Aluru and Vijayan, 2009). During salinity challenge, more level of cortisol needs to compensate the energy to osmoregulation and osmotic stress as increase chloride cell number and size (McCormick, 1990) and increase in Na^+,K^+-ATPase activity (Madsen, 1990). In the present study, the cortisol elevation was occurred 24 and 72 h after saltwater exposure. This result is agree with Ghelichpour et al. (2018) which common carp exposed to indoxacarb. Glycogen utilization leads to glucose production in fish liver, thus, increased circulating glucose levels. As glycogen stores are limited, prolonged stress leads to gluconeogenesis from lactate and amino acids to maintain hyperglycemia (Barton, 2002). In the present study, the pesticide groups had significantly higher glucose compared to the control, suggesting induction of stress by these pesticides. Similarly, Taheri Mirghaed et al. (2018) found increase in plasma glucose due to indoxacarb exposure in common carp. Simakani et al. (2018) found hyperglycemia in common carp after exposure to mancozeb. On the other hand, exposure to saltwater led to increase plasma glucose. Similarly, Ghelichpour et al. (2018) and Hoseini and Hosseini (2010) reported increased plasma glucose in common carp exposed to saltwater.

Sodium, potassium and chloride are the most abundant ions in fish blood with varieties of vital roles (Bonga and Lock, 1991). In the present study, saltwater exposure led to significant elevation in plasma sodium and chloride in common carp. It is in line with previous studies showing such elevations in plasma sodium and chloride in common carp exposed to saltwater (Ghelichpour et al., 2018; Taheri Mirghaed et al., 2018). Potassium level of plasma increased in pesticide groups and after saltwater exposure, it showed fluctuation. Common carp is a stenohaline species and cannot tolerate wide range of water salinity, instead, it augment internal ions levels to mitigate osmotic gradient across body surface and prevention of water lose (Van der Linden et al., 2004). Exposure to lufenuron and flonicamid significantly decreased plasma chloride. It has been previously reported that pesticides decrease chloride levels via two ways: 1) induction of stress and increase in kidney

chloride loss, and 2) effects on carbonic anhydrase activity in fish gill (Taheri Mirghaed et al., 2018). Similarly, Banaee et al. (2014) found decrease in plasma chloride in common carp exposed to chlorpyrifos.

Calcium is an important ion in fish circulation, which its levels is tightly controlled in healthy fish (Kaneko and Hirano, 1993). In this study, pesticide exposure modified calcium responses to saltwater exposure; the control group showed no changes in plasma calcium levels during saltwater exposure, but the pesticide groups had elevated calcium levels within this period. Such changes might be due to stress induced by pesticides that leads to cortisol elevation and induction of calcium pump in fish (Hoseini et al., 2018). On the other hand, it has been reported that saltwater exposure leads to increased calcium (Jalali et al., 2010). Higher changes in calcium in the pesticide groups might be due to gill damages, impairing osmoregulation and ionoregulation during saltwater exposure. Phosphorus level increased 24 h after salinity challenge and then decreased which the same as pre salinity challenge.

Conclusion

In conclusion, the results demonstrate that sub-lethal exposure to lufenuron and flonicamid impairs cortisol and glucose response to saltwater stress. It interferes glucose metabolism and hydromineral balance. All these effects threatens the fish life chance in response to saltwater stress.

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