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## Cytotoxic Effect of Cypermethrin on *Pimpla turionellae* (Hymenoptera: Ichneumonidae) Larval Hemocytes

Fevzi UCKAN<sup>1</sup>, Olga SAK<sup>2</sup>

<sup>1</sup> Department of Biology, Faculty of Science-Literature, Kocaeli University, 41300, Kocaeli-TURKEY

<sup>2</sup> Department of Biology, Faculty of Science-Literature, Balıkesir University, 10145 Balıkesir-TURKEY

\*Corresponding author: olgasak@hotmail.com

### Abstract

The effects of cypermethrin on the hemocytes of pupal endoparasitoid *Pimpla turionellae* (Hymenoptera: Ichneumonidae) feeding on its host *Galleria mellonella* (Lepidoptera: Pyralidae) was evaluated after treatment of the host larvae with different sub-lethal doses of the insecticide. Four hemocyte types; prohemocytes, plasmatocytes, granulocytes, and adipohemocytes were identified in parasitoid last instars hemolymph, based on morphological criteria. Dose-dependent changes in the frequencies of mitosis, apoptosis, and micronuclei in hemocytes were determined and compared. Cypermethrin exposure reduced the mitotic activity yet increased the apoptosis in a dose-dependent manner. Mean frequencies per dose in the hemocytes decreased from 27 to 1‰ and increased from 22 to 113‰ for mitosis and apoptosis, respectively. Micronucleated cells were rare but their frequency increased in a dose-dependent manner. The decrease in mitosis along with the rapid increase in apoptosis in wasp hemocytes suggests that parasitoids may consume more energy and their defense mechanism may be destroyed due to the depletion in the hemocyte number.

**Keywords:** Apoptosis, insecticide, micronucleus, mitosis, parasitoid.

### Cypermethrinin *Pimpla turionellae* (Hymenoptera: Ichneumonidae) Larvalarında Hemositler Üzerine Sitotoksik Etkisi

#### Özet

Konak tür, *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvalarına farklı dozlarda besin içinde verilen cypermethrinin pup parazitoiti, *Pimpla turionellae* (Hymenoptera: Ichneumonidae) hemositlerine etkileri araştırıldı. Parazitoit son evre larva hemolenfinde morfolojik özelliklerine göre prohemosit, plazmatosit, granülosit ve adipohemosit olmak üzere dört hemosit tipi tanımlandı. Cypermethrin dozuna bağlı olarak hemositlerin mitoz, apoptozis ve mikroçekirdek değerlerinde görülen değişiklikler belirlendi. Cypermethrin uygulaması doza bağlı olarak mitotik aktivitede azalma (‰27 den ‰1'e kadar), apoptotik hücre sayısında ise artmaya (‰22'den ‰113'e kadar) neden oldu. Mikroçekirdek görülen hücre sayısı az olmakla beraber mikroçekirdekli hücre sayısı doza bağlı olarak artış gösterdi. Mitoz oranı düşerken apoptotik hücre ölümünün artması parazitoitin fazladan enerji harcamasına, hemosit sayısındaki düşme ise savunma mekanizmasının zarar görmesine neden olacaktır.

**Anahtar Kelimeler:** Apoptozis, insektisit, mikroçekirdek, mitoz, parazitoit.

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### INTRODUCTION

The use of natural enemies with insecticides in depressing the populations of pests has recently justified studies to determine the harmful effects of insecticides on nontarget species (Nowak et al. 2001). The assessment of the potential effects of insecticides on parasitoids is important for the success of an effective Integrated Pest Management (IPM) programs. Several studies have shown that many sublethal doses of insecticides limit the development, survival and growth of parasitoid

wasps either by direct chemical contact or by ingestion of treated prey (Simmonds et al. 2002, Sak et al. 2006, Ergin et al. 2007).

The possible involvement of insect hemocytes in curbing the biological and chemical control efforts of pests is also apparent due to their implication in the detoxification of insecticides (Lackie 1988). Multifunctional roles of hemocytes such as phagocytosis, encapsulation, nodule formation, cell agglutination, detoxification as well as synthesis, and secretion of the humoral immunity factors may

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render them more sensitive than other cells towards internal and external factors. Consequently, these cells are frequently used to ascertain the cytogenetic damage of toxic chemicals (Begum and Gohain 1996, Gayfullina et al. 2006). A number of toxicological studies have examined the effects of insecticides on the chemical changes in insect hemolymph (Nath et al. 1997, Serebrov et al. 2001). However, information about the physiology of hemocytes in insects that have been exposed to insecticides is meager (Jones 1962).

*Pimpla turionellae* (L.) (Hymenoptera: Ichneumonidae) is a polyphagous endoparasitoid that spends its immature stages in pupae of various lepidopterous species. It serves as a potential biological control agent of many lepidopterous pests (Kansu and Uğur 1984, Fisher 1987). Adult wasps feed on plant nectar and host pupae in nature. Therefore, it is possible for parasitoid wasps to be exposed to insecticide by way of feeding and contact. We have previously reported the effects of cypermethrin on the total body weight, glycogen, protein, and lipid contents of *P. turionellae* during development (Sak et al. 2006). Cypermethrin [CYM, ( $\pm$ )  $\alpha$ -cyano-3-phenoxybenzyl ( $\pm$ ) cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] is a non-systemic pyrethroid insecticide with contact and stomach action and has broad-spectrum use in worldwide (Kamrin 1997). Besides killing target pests, it can also reduce populations of insects that are economically desirable such as parasitoids, predators, bees, etc. (Cox 1996, Kamrin 1997). Here, we aimed to show how cypermethrin is likely to be accumulated in the host, affects the cytological aspects of hemocytes from parasitoid last instars. We also characterized the main types of hemocytes from *P. turionellae* based on morphological criteria.

#### MATERIAL AND METHODS

**Insect rearing:** Insects were cultured as previously described (Sak et al. 2006). Briefly, the pupal endoparasitoid *P. turionellae* was reared in the laboratory on the pupae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) at  $25 \pm 1^\circ\text{C}$ ,  $60 \pm 5\%$  RH and a photoperiod of 12 hrs light and 12 hrs dark. The adult parasitoids were fed a 30% honey solution and provided with host pupae (4 pupae for 10 wasps). The host colony was maintained by feeding the insects with a diet described by Bronskill (1961) and modified by Sak et al. (2006).

**Insecticide application:** Cypermethrin (Impre-tor, 250 g/liter EC, Zeneca Ltd., İzmir, Turkey) was prepared in distilled water as parts per million of the active ingredient and added to the diet in all bioassays. The median pupation dose (PD<sub>50</sub>) value of *G. mellonella* exposed to cypermethrin was previously determined as 207.3 (181.7 - 235.1) ppm (Sak et al. 2006). Hence, we applied doses below PD<sub>50</sub> to the host last instars to evaluate the dose-dependent effect of cypermethrin on hemocytes of *P. turionellae* last instars. Various doses (20, 50, 100, and 150 ppm) of cypermethrin and a distilled water control were incorporated into the artificial diet of the host larvae. Batches of 50 host larvae ( $0.16 \pm 0.01$  g) were exposed to 50 g of the diet including the selected doses of cypermethrin for seven days. The jars were maintained in another laboratory under the same conditions mentioned for the stock cultures. Larvae were removed from the diet and those pupated were parasitized by *P. turionellae* females. Parasitoid last instars that had progressed through 5 days of the final larval stadium were isolated from the host pupae and used in the hemocyte studies.

**Isolation and characterization of hemocytes:** The *P. turionellae* last instars were washed in distilled water and were blot dried on sterile tissue paper at room temperature. The larvae were bled by folding their bodies in half gently and puncturing the ventral integument with an insect pin at the fold. The hemolymph was immediately bled directly on a glass slide, overspread, and allowed to dry at room temperature for 10 to 20 min. During this time the hemocytes adhered to the glass. The cells were then fixed in glacial acetic acid-methanol (1:3 vol: vol) (Merck) for 10 min. After natural air-drying of the fixative, the hemocytes were stained with 5% Giemsa (Merck) for 5-6 min. The slides were then rapidly washed with distilled water and buffer solutions. After air drying, the slides were dehydrated with xylene (Merck) and mounted in Entellan (Merck). The hemocytes were examined and visualized with an Olympus BX51 microscope equipped with a digital camera Olympus C-4000 200M. The hemocyte types were identified by using established morphological characters presented by Jones (1962) and Gupta (1985). The average size of the hemocyte types were estimated by measuring the length and width of twenty cells of each type.

**Effects of cypermethrin on hemocytes:** The

effects of cypermethrin on the hemocytes of *P. turionellae* larvae were examined under 1000x magnification. The micronucleated and apoptotic cells were identified following generally accepted criteria: nucleus particles not touching the main nucleus and smaller in size in comparison to the main nucleus were evaluated as micronuclei (MN) (Venier et al. 1997). The characteristics of the apoptotic hemocytes included cell surface blebbing and condensation of chromatin (Pech and Strand 2000). Binucleated cells were also detected independently when present and the mitotic figures of hemocytes were carefully identified. All experiments were replicated four times with one larva per replicate for each dose point of the different treatments. At least 1,000 cells per larva and 4,000 cells per dose point were analyzed for mitotic, apoptotic, and micronucleated hemocytes.

The data was subjected to linear regression analysis (SPSS, 10.0, SPSS Science, Chicago, IL) to determine the responses of the different cytological measures to the insecticide doses. Relationships between cypermethrin doses and cytological measures (mitosis, apoptosis, and micronuclei in hemocytes) were developed using data calculated from each replicate and fitted to the equations  $y = a + bx$  using linear regression where  $y = \log$ mitosis, apoptosis, and micronuclei, respectively and  $x =$  cypermethrin dose. The results were considered statistically significant when  $P < 0.05$ . The patterns of dose related changes in cytological measures were examined graphically by plotting the rate of mitosis, apoptosis, and micronuclei against the cypermethrin doses.

## RESULTS

**Hemocyte types:** Four well-defined hemocyte types could be distinguished in the hemolymph of the parasitoid larvae: prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), and adipohemocytes (ADs) (Fig. 1). The prohemocytes and plasmatocytes were the most common hemocyte types. The prohemocytes were small, round or slightly oval cells [ $8.20 \pm 0.43$  (SE)  $\mu\text{m}$  long and  $7.45 \pm 0.41$   $\mu\text{m}$  wide,  $n = 20$ ], with a thin rim of cytoplasm surrounding the nucleus (Fig. 1A). In the Giemsa stain, the cells were intensely basophilic. The plasmatocytes were highly polymorphic cells with variable sizes; they showed spherical ( $13.95 \pm 0.49$   $\mu\text{m}$  long and  $12.9 \pm 0.52$   $\mu\text{m}$  wide,  $n=20$ ), oval ( $17.85 \pm 0.6$   $\mu\text{m}$  and  $11.0 \pm 0.5$   $\mu\text{m}$ ), elongated or

spindle-shaped ( $36.2 \pm 2.27$   $\mu\text{m}$  and  $10.38 \pm 0.49$   $\mu\text{m}$ ) form, when viewed under a light microscope (Fig. 1B-C). The nuclei were round or elongated, and generally centrally located.

The majority of the PLs were mononucleated, but some binucleated cells were also observed (Fig. 1C). After the Giemsa staining, the PLs showed a variable number of granules that were negatively or positively stained. The PLs and PRs presenting same shape could be distinguished from each other by the higher nuclear/cytoplasmic ratio in the later. PLs contained fewer granules than the GRs.

Granulocytes were spherical or oval cells with variable sizes ( $15.65 \pm 0.83$   $\mu\text{m}$  long and  $13.75 \pm 0.69$   $\mu\text{m}$  wide,  $n = 20$ ). The cytoplasm of GR contained numerous small and highly basophilic granules (Fig. 1D). The spherical and oval GRs presented round or slightly elongated nuclei that were generally centrally located.

The adipohemocytes were also polymorphic, with spherical or oval cells with variable sizes ( $13.9 \pm 0.7$   $\mu\text{m}$  long and  $11.73 \pm 0.62$   $\mu\text{m}$  wide,  $n=20$ ). The nuclei were relatively small (compared to those of PLs or GRs), round or slightly elongated, and centrally or eccentrically located. The cytoplasm contained characteristic large refringent lipid-like inclusions, which sometimes obscured the nuclei contour, and appeared prominent on the cell surface (Fig. 1E-F).

**Effects of cypermethrin on hemocytes:** The parameters of the equations describing the effects of cypermethrin doses on mitosis, apoptosis, and micronuclei of hemocytes from wasps fed on cypermethrin-treated hosts are presented in Table 1. There appeared significant dose effects on the mitosis and apoptosis but variations among doses were not considerably different with micronuclei (Table 1). The patterns of mitosis, apoptosis, and micronuclei as a function of cypermethrin doses are illustrated in Figs. 2-4. Interestingly, the effect of apoptosis was linear whereas the effect on mitosis appeared to be logarithmic. Despite the insignificant variability evident in micronucleated hemocytes ( $P > 0.05$ ), a gradual dose-dependent decrease in mitosis ( $P < 0.005$ ) and an increase in apoptosis ( $P < 0.001$ ) with respect to controls were detected in the hemocytes of wasps exposed to cypermethrin. In fact, across the doses, the mean frequencies decreased from 27 to 1‰ and increased from 22 to 113‰ for hemocytes demonstrating mitosis and

apoptosis, respectively. The mitotic activities of hemocytes were not significantly different from each other across the different doses of insecticide whereas, the apoptotic frequency was by far the highest at 150 ppm. Mitotic, apoptotic and micronucleated hemocytes from *P. turionellae* last instars are shown in Fig. 5.

### DISCUSSION

The major result of this research is that feeding by a host on a sub-lethal cypermethrin-treated diet cytotoxicologically affected the wasp hemocytes. This result is consistent with our previous results that demonstrated cypermethrin affected total body weight and storages of glycogen, protein, and lipid in *P. turionellae* (Sak et al. 2006). Previously we had also determined cypermethrin induced changes in the egg to adult development time, number of offspring produced, and longevity of the larval endoparasitoid *Apanteles galleriae* Wilkinson (Hymenoptera: Braconidae) (Ergin et al. 2007).

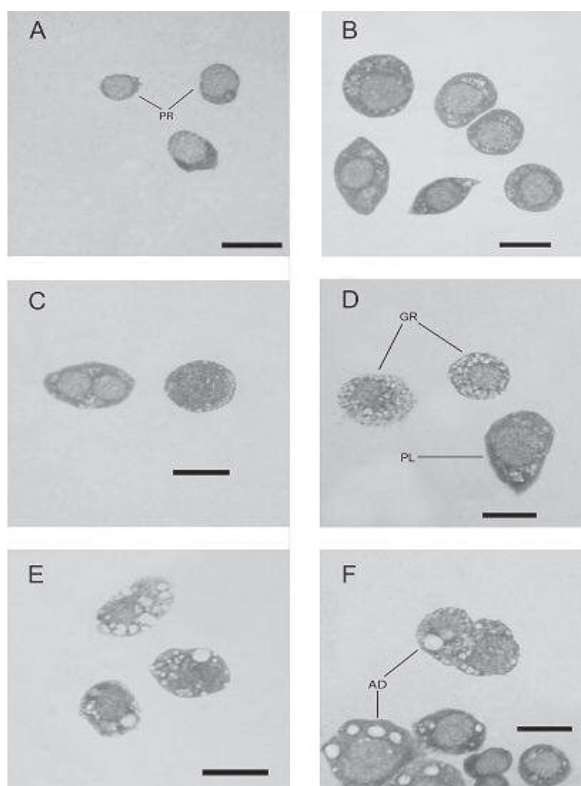
Our previous and current results support the idea that environmental pollutants are more hazardous for parasitoids than for their hosts either due to their potential for bioaccumulation and/or due to a greater susceptibility to pollutants than their hosts (Ortel 1995). It is likely that changes in development, growth, mortality, reproduction and in concentration of the storage compounds in the parasitoids are caused by an alteration in the contaminated host hemolymph composition (Gintenreiter et al. 1993, Ortel et al. 1993, Bischof 1995). In this study only the hosts were exposed to the pesticide and parasitoids were indirectly exposed through host hemolymph.

Following the described procedure and Giemsa staining, we distinguished four cell types in the hemolymph of *P. turionellae* larvae as prohemocytes, plasmatocytes, granulocytes, and adipoheocytes. Gupta (1985) describes seven basic types of hemocytes; four of them which we also identified in the present study plus spherulocytes, oenocytoids, and coagulocytes. The utilized methodology and staining conditions did not allow further discrimination. Histochemical characterization or electron microscopy is needed to distinguish the other different cell types. In spite of the difficulty to compare the hemocytes among the different species, the data already published and the morphology described here for *P. turionellae* allowed us to make this comparison. The main features used to identify

**Table 1.** The parameter values ( $\pm$  SEM) for equations describing the relationship between cypermethrin doses and mitosis, apoptosis, and micronuclei of hemocytes from *P. turionellae* fed on cypermethrin-treated *G. mellonella*.

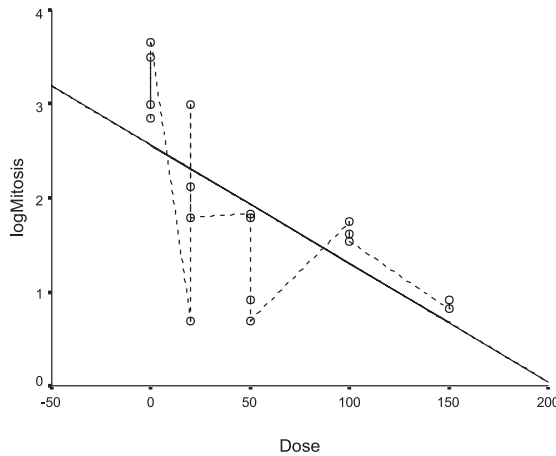
Response variables	Model parameters					
	<i>a</i>	<i>b</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>r</i> <sup>2</sup>
Mitosis	2.559 (0.27)	- 0.013 (0.00)	11.158	1, 15	0.004	0.43
Apoptosis	22.248 (5.25)	0.511 (0.62)	67.154	1, 18	0.000	0.79
Micronuclei	0.127 (0.41)	0.006 (0.005)	1.684	1, 18	0.211	0.09

Numbers in parenthesis are standard errors of the estimates.

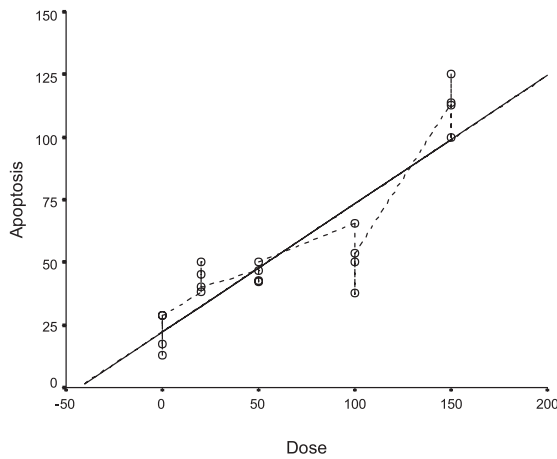


**Fig 1.** Light micrographs of hemocytes from the last instars of *P. turionellae*. (A) Prohemocytes. (B) Spherical and elongated plasmatocytes. (C) Binucleated and spherical plasmatocytes. (D) Granulocytes and plasmatocyte. (E-F) Adipoheocytes. Photographs were made using a 100x objective. The bars correspond to 10  $\mu$ m.

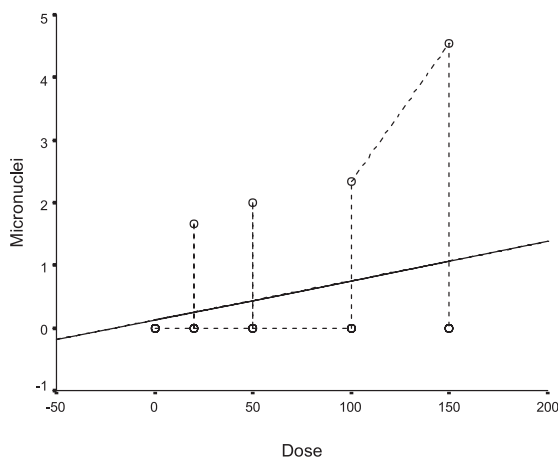
*P. turionellae* hemocytes by light microscopy were size, the presence of granules in the cytoplasm, and staining type of the cells. Prohemocytes were smaller and had a large nucleus-cytoplasm ratio. Consequently, prohemocytes of *P. turionellae* certainly correspond to the prohemocytes described by several authors for other insects (Cebesoy and Ayvalı 1996, Rivers et al. 2002, Silva et al. 2002). Plasmatocytes observed in the hemolymph smears of *P. turionellae* were the most variable cells in shape. They were spherical, oval, elongated or spindle-



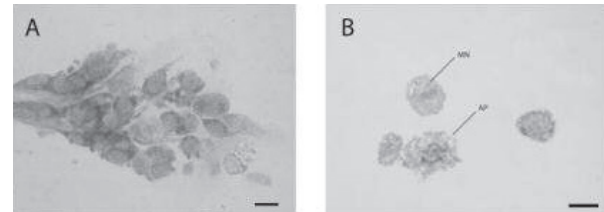
**Fig 2.** The relationship between the mitosis and the cypermethrin doses.



**Fig 3.** The relationship between the apoptosis and the cypermethrin doses.



**Fig 4.** The relationship between the micronuclei and the cypermethrin doses.



**Fig 5.** Light micrographs of hemocytes from the last instars of *P. turionellae*. (A) Hemocytes undergoing cell division. (B) Examples of apoptotic and micronucleated hemocytes. Photographs were made using a 100x objective. The bars correspond to 10  $\mu$ m.

shaped. Our observation that plasmatocytes are polymorphic cells is in agreement with those of Silva et al. (2002) and Hernandez et al. (1999). Granulocytes of *P. turionellae* appeared to correspond to the granulocytes of other insects reported (Beeman et al. 1983, Silva et al. 2002, Ribeiro and Brehélin 2006). They could be easily identified by their cytoplasm characteristically filled with basophilic granules in giemsa-stained smears. Adipohemocytes in *P. turionellae* were exhibiting a large lipid-like vesicle. Their characteristics are also similar to those observed in other insect species (Gupta 1985, Silva et al. 2002).

During development, it is a prerequisite that prodigious numbers of cells die to facilitate normal differentiation and homeostasis (Dai and Gilbert 1997, Jones and Schwartz 2001). Here, we report a logarithmic decrease exists between the levels of mitosis and cypermethrin doses. On the other hand, dose-related increases in apoptotic indices were linear. It is likely that as a defensive mechanism parasitoids primarily retard mitosis and undergo apoptosis later. However, cell death by apoptosis is an active process that involves activation of specific genes and ATP-dependent cellular energy (Jones and Schwartz 2001). The decrease in mitosis along with the increase in apoptosis in wasp hemocytes suggests that parasitoids may consume more energy and their defence mechanism may be destroyed due to the depletion in hemocyte numbers. This is consistent with our previous analysis suggesting that *P. turionellae* larvae display a significant decrease in their lipid storage when exposed to cypermethrin (Sak et al. 2006). Increasing energy demand may result from homeostatic processes such as detoxification (Choi et al. 2001).

None of the hemocytes showed MN whereas high mitotic activity was detected in the hemocytes

of control group. When MN values obtained at each cypermethrin dose were compared to that of the control, there were no significant differences. Micronucleated cells were also rare in the slides obtained from the hemocytes of the exposed wasps although it seemed MN frequency increased in a dose-dependent manner. High variability has already been observed in the MN frequency of mussel hemocytes exposed to different pollutants (Venier et al. 1997, Pinto-Silva et al. 2003). The lack of micronucleated hemocytes in this work did not allow us to evaluate cytotoxicity of cypermethrin along with mitosis and apoptosis for *P. turionellae*.

Future in-depth study with higher doses of cypermethrin is necessary. However, the fact that cypermethrin induced apoptosis while suppressing mitosis in hemocytes along with recent work reporting decreases in protein and lipid storages of wasp larvae (Sak et al. 2006) argues for the possible concurrent cytotoxic and physiological effects of the pyrethroid.

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