Apidologie 35 (2004) 453–460
© INRA/DIB-AGIB/ EDP Sciences, 2004
DOI: 10.1051/apido:2004037

Original article

Cell death in honeybee (*Apis mellifera*) larvae treated with oxalic or formic acid

Aleš GREGORC[a]*, Azra POGAČNIK[a], Ivor D. BOWEN[b]

[a] Veterinary Faculty of the University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia
[b] Cardiff School of Biosciences, Preclinical Building, Cardiff University, PO Box 911, Cardiff, CF10 3US, UK

(Received 3 June; revised 28 November 2003; accepted 16 December 2003)

Abstract – The effects of oxalic (OA) and formic acids (FA) on honeybee larvae in colonies were assessed and evaluated. Cell death was detected by the TUNEL technique for DNA labelling. In 3- and 5-day-old larvae exposed to OA, cell death was found in 25% of midgut epithelial cells 5 h after the treatment, using an "In situ cell death detection kit, AP" (Roche). The level of cell death increased to 70% by the 21st hour and the morphology of the epithelium remained unchanged. Fifty hours after the application, cell death was established in 18% of the epithelial cells of the 3-day-old larvae and had increased to 82% in the 5-day-old larvae. A "DeadEnd" apoptosis detection kit (Promega) showed sporadic cell death mainly in the larval fat body 5 h after treatment. Twenty-one hours after the OA application cell death was found in 4% of the larval midgut epithelial cells. Evaporated formic acid induced extensive apoptotic cell death in the peripheral, cuticular and subcuticular tissues that preceded the cell death of the entire larval body.

***Apis mellifera* / cell death / oxalic acid / formic acid / immunochemical method**

1. INTRODUCTION

Cell death can occur either by accident, referred to as necrosis, or by design, which is described as programmed cell death or apoptosis (Bowen et al., 1996). It has been shown that apoptosis can be induced by genetic or by non-genetic means (Ellis et al., 1991; Arends and Wyllie, 1991). Necrotic cell death appears to be induced under extreme conditions such as ischaemia, hypoxia, exposure to toxins and hyperthermia (Bowen et al., 1996). Necrosis also refers to the post-mortem changes that occur following the death of the cell (Trump and Berezesky, 1998). Cell death has been revealed in the regressive hypopharyngeal glands of worker honeybees (Moraes and Bowen, 2000), in the honeybee midgut after a *Paenibacillus larvae* infection and acaricide amitraz application (Gregorc and Bowen, 1998, 2000), and in the isolated atria of the heart of the honeybee (*Apis mellifera*) after exposure to the herbicide 2,4-Dichlorophenoxyacetic acid (Papaefthimiou et al., 2002).

The DNA breakdown that precedes the nuclear collapse of apoptotic nuclei can be tested using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling, which is generally termed a TUNEL assay (Levy et al., 1998; Sgonc and Gruber, 1998). In our previous experiments (Gregorc and Bowen, 2000), higher cell levels were found when an "In situ cell death detection kit, AP" (Roche) was used as compared to an ApopTag kit (Oncor). The "In situ cell death detection kit, AP" was unable to differentiate between apoptosis

* Corresponding author: ales.gregorc@vf.uni-lj.si

and accidental cell death in different tissues and detected both necrosis and apoptosis (Matylevitch et al., 1998). The ApopTag kit (Oncor) labelled only DNA-fragmented apoptotic cells (Short et al., 1997). Thus, DNA fragmentation and a TUNEL-positive reaction can appear after different kinds of cell death using various kits (Orita et al., 1999; Grasl-Kraupp et al., 1995). The Promega Apoptosis detection system measures nuclear DNA fragmentation as a specific indicator of apoptosis (Sperandio et al., 2000).

Honeybee larvae can experience various chemical stresses. The chemicals that are most often encountered are acaricides against the *Varroa destructor* parasite. The aim of our work was to determine whether different treatments of honeybee larvae with "alternative" Varroa control substances such as oxalic and formic acid cause cell death, which can be detected using the TUNEL method. In this respect we have therefore attempted to study the distribution of cell death in treated honeybee larvae.

2. MATERIALS AND METHODS

In this study *Apis mellifera* L. larvae were treated in the comb with either oxalic or formic acid. The larvae were treated with an oxalic acid (OA)/sucrose solution, prepared from 6.5 g oxalic acid dihydrate (Kemika, Zagreb, Croatia) and 50 g sucrose in 100 mL deionized water. The concentration of OA/sucrose was 2.97%/31.95% (w/w). The substance was sprayed directly on the larvae in the comb cells so that each treated larva received approximately 0.121 mg of the OA solution. Combs with treated larvae were placed back into the colony. The larval age was established by marking the comb cells at the egg stage, on a transparent foil, one day after oviposition.

Formic acid was employed in a concentration of 85% in deionized water. An evaporator, produced in Slovenia by the Medja company, was used for applying the FA in the honeybee colony. It consisted of a transparent tank with a millilitre scale and an opening on the top, in which a softboard strip was inserted. The softboard was positioned so that one side of it was in contact with the formic acid, which allowed it to soak through the strip and evaporate outside the tank from the other side. It was placed approximately 10 cm (± 3) from the brood. The quantity of evaporated-FA solution in the hive was approximately 10 mL (± 2.5) per day. Nurse bees fed the larvae and removed any that had died. The larvae were sampled 5, 21 and 50 h after the application of the acids. Control untreated larvae were also collected at the same time intervals. Three larvae of different ages from the experimental groups were sampled and separately prepared for light immunohistochemistry.

The sampled larvae were dissected into three parts, in order to improve fixative penetration inside the larval part containing the midgut tissue, and fixed for 24 h in a 10% formol saline. They were then dehydrated in a series of alcohols and xylene and finally embedded in wax as described by Gregorc and Bowen (1999). A Bright 5030 microtome was used to cut 5 μm sections, which were placed on Decon-cleaned slides and then air-dried.

The slides were washed three times in xylene and three times in absolute alcohol to remove paraffin wax from the tissue sections. Other procedures were conducted in accordance with the test kit instructions. Two kits were used in order to establish the cell death phenomenon in the bee larvae after the formic or oxalic acid applications and to determine the eventual differences in the levels of cell death.

Using the "In situ cell death detection kit, AP" (ISCDDK) (Roche), dewaxed and rehydrated tissue sections were incubated with proteinase K for 15 min. Labelling was conducted by covering the tissue sections with 30–40 μL of a "TUNEL" reaction mixture, composed of terminal deoxynucleotidyl transferase (TdT) from calf thymus, for 60 min at 37 °C in a humidified chamber. Any TdT-enzyme-incorporated fluorescein was detected with a "converter-AP" consisting of an anti-fluorescein antibody from sheep and conjugated with alkaline phosphatase, which was included in the kit. The EnVision System alkaline phosphatase kit (Dako), which contained an alkaline-phosphatase labelled polymer and a fast red chromogen solution, was used to obtain a red-coloured precipitate. The sections were counterstained with haematoxylin. TUNEL-positive cells appeared red, whereas TUNEL-negative nuclei appeared blue. Negative-control labelling was achieved by substituting the deoxynucleotidyl transferase (TdT) enzyme with PBS.

The DeadEnd colorimetric apoptosis detection system (Promega) labels fragmented DNA of apoptotic cells in situ using TUNEL assay. After applying proteinase K, the sections were incubated with the TdT reaction mixture and then with a horseradish-peroxidase-labelled streptavidin solution. DAB (diaminobenzidine) substrate was then applied onto the tissue sections to develop a brown reaction product. The sections were counterstained with methyl green. Negative control labelling was achieved by substituting the deoxynucleotidyl transferase (TdT) enzyme with PBS.

In order to establish the percentage of cells affected by the various treatments, at least 100 cells

Table I. Quantified values of cell death in honeybee larvae midgut-columnar cells obtained after oxalic (OA) or formic (FA) acid treatments as mean percentages and SD of counts on at least 100 cells from 3 samples. Two standard detection kits were used: the "In situ cell death detection kit, AP" (ISCDDK) and the "DeadEnd colorimetric apoptosis detection system" (Promega). Data showing the effect of FA application to 3- and 5-day-old larvae using DeadEnd were obtained from counting the columnar midgut-epithelial cells and, as indicated in the table, cells in the haemocoel. Samples were collected 5 h, 21 h and 50 h after each acid application.

		ISCDDK			DeadEnd		
		5 h	21 h	50 h	5 h	21 h	50 h
OA	3-day-old l.	23.3 ± 4.7	76.3 ± 4.0	18.2 ± 7.7	3.3 ± 2.0		3.3 ± 1.5
	5-day-old l.	26.3 ± 2.0	66.3 ± 8.5	81.6 ± 4.1		4 ± 1.0	
FA	3-day-old l.	69.6 ± 32.8	96.3 ± 3.2			2.7 ± 2.0	
	5-day-old l.	95 ± 3.0	100 ± 0.0			Cells in haemocoel	
						47 ± 8.0	98.3 ± 2.8
Control	3- and 5-day-old l.	2.6 ± 2.0	4.3 ± 1.1			1.6 ± 2.0	

were scored in random fields for TUNEL labelling using tissue from the three examined larvae samples. The midgut and surrounding tissue, together with the larval cuticula, were examined and the cell death evaluated. The mean ± SD of the TUNEL-labelled cells was calculated as a percentage. Light microscopy of the midgut epithelial cells and photography were conducted using a Nikon light microscope.

3. RESULTS

3.1. In situ cell death detection kit, AP (ISCDDK)

Five hours after the honeybee larvae were exposed to OA, a red azo-dye reaction product was found in 25% of the midgut columnar-cell nuclei and none of the midgut regenerative-epithelial cells reacted positively (Fig. 1). Twenty-one hours after the OA treatment, the level of positive-reaction product in the columnar midgut cells (Fig. 2) had risen to approximately 76% in both the 3- and 5-day-old larvae, while there was still no reaction found in the regenerative midgut epithelial cells (Tab. I). In comparison with the control, water-treated larvae the general morphology of the epithelium was unchanged. The reaction product was localised in 18% of the columnar-epithelial cells of the 3-day-old larvae 50 h after the OA application and the level of positive midgut cells of the 5-day-old larvae reached to 81%. The reaction product was found sporadically in the regenerative midgut cells (Figs. 3a, b).

In the FA-treated larvae a reaction product was found in the nuclei of the columnar midgut-epithelial cells though not in the regenerative cells. There appeared to be an increase in the degree of vacuolation and granulation of the cytoplasm (Fig. 4). The levels of cell death in the treated larvae ranged between 70 and 100% and these details are shown in Table I.

3.2. The DeadEnd colorimetric apoptosis detection system

The Promega DeadEnd kit gives a brown reaction product. Sporadic DAB-positive cells were found in the OA-treated larvae 5 h after treatment. Positive cells were mainly found in the larval fat body (Fig. 5a). Twenty-one hours after the OA application, DAB-positive cells were found in approximately 4% of the larval midgut extruded-epithelial cells (Fig. 5b, Tab. I).

In the FA-treated larvae, a DAB-reaction product was found in the peripheral, cuticular and subcuticular tissues. Twenty-one hours after the FA treatment, the DAB-reaction product was localised in the cuticular cells, subcuticular cell nuclei and in the cytoplasm of the fat and glandular cells of the haemocoel (Fig. 6a). There was no localised reaction product in the untreated control larvae. Fifty hours after the FA treatment, the DAB-reaction product was localised in all the tissues of the larval body excluding the midgut-epithelial cells, where the DAB-reaction product was found only sporadically (Fig. 6b).

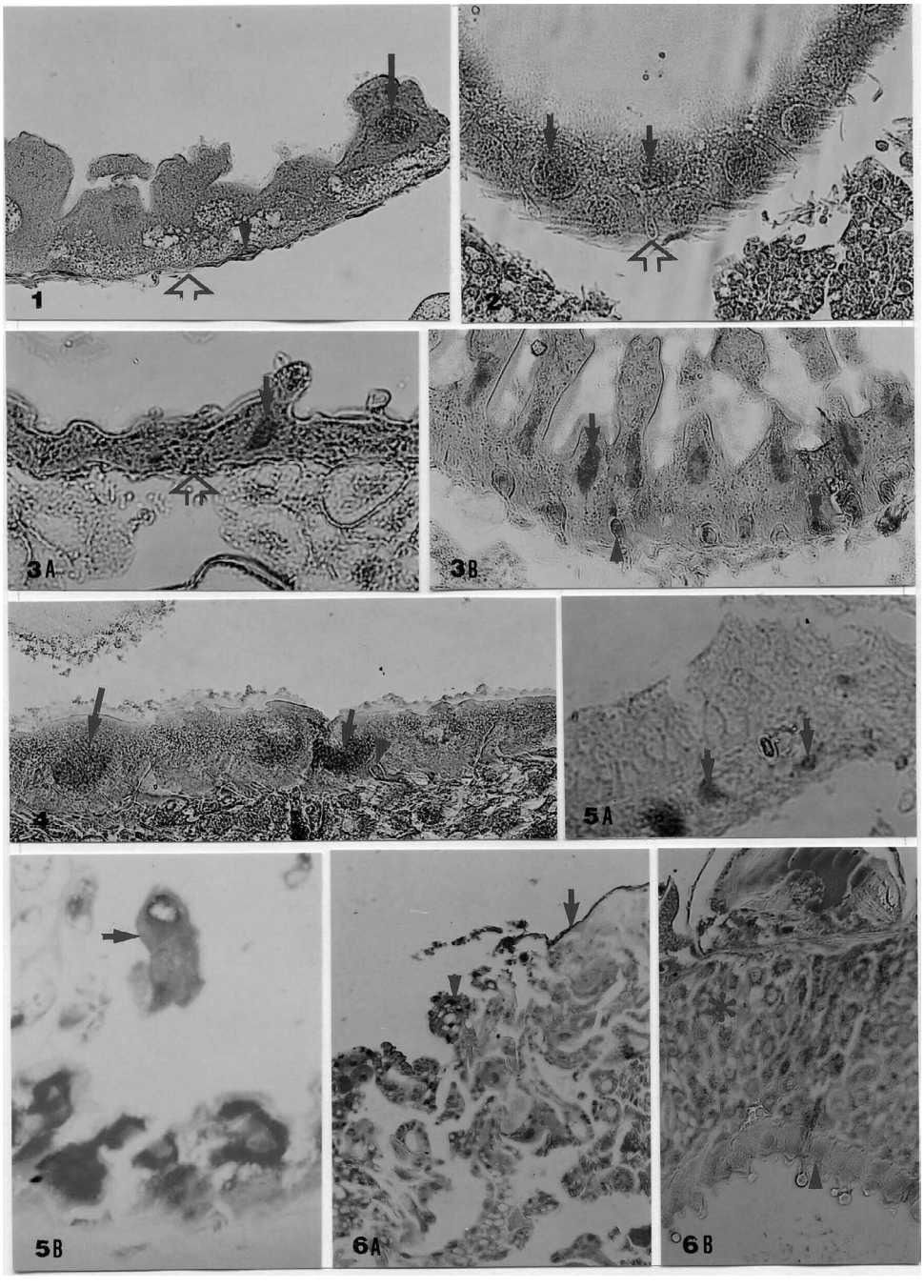
1
2
3A
3B
4
5A
5B
6A
6B

Figure 1. Midgut epithelial cells of formalin-fixed, paraffin-embedded 5-day-old larvae, 5 h after an oxalic acid (OA) treatment. Cell death was detected by the TUNEL technique using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP for DNA labelling, an anti-fluorescein alkaline phosphatase conjugated antibody (ISCDDK), fast red for visualization, and counterstaining with haematoxylin. Dense red azo dye staining localised to the nuclei (➡) of the columnar cells of the midgut epithelium. Negative regenerative cells (▼). Epithelial basal lamina (⇨). Magnification: 200 x.

Figure 2. Staining of formalin-fixed, paraffin-embedded larvae and the TUNEL technique was performed using "In situ" cell death detection kit (Roche). The figure shows 3-day-old larvae, 21 h after an OA treatment. Red azo dye staining is localised in the midgut columnar epithelial-cell nuclei (➡) indicative of impending cell death. Staining is absent in the regenerative epithelial-cell nuclei (⇨). Magnification: 200 x.

Figure 3. Midgut epithelial cells of formalin-fixed, paraffin-embedded larvae, 50 h after an OA treatment. Cell death was detected by the TUNEL technique using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP for DNA labelling, an anti-fluorescein alkaline phosphatase conjugated antibody (ISCDDK). Panel A shows sections of the midgut of 3-day-old larvae. Alkaline phosphatase is localised to the nuclei of columnar epithelial cells (➡) and sporadic staining of the nuclei of regenerative epithelial cells (⇨). Magnification: 200 x. Panel B shows red azo dye staining localised to the nuclei of columnar (➡) and sporadic regenerative epithelial cells (▼). Magnification: 200 x.

Figure 4. Midgut epithelial cells of formalin-fixed, paraffin-embedded 3-day-old larvae, 21 h after a formic acid (FA) treatment. Cell death was detected by the TUNEL technique using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP for DNA labelling, an anti-fluorescein alkaline phosphatase conjugated antibody (ISCDDK). Alkaline phosphatase is localised to the nuclei of columnar epithelial cells (➡) and was not localised to the nuclei of regenerative epithelial cells (▼). Magnification: 400 x.

Figure 5. Staining of formalin-fixed, paraffin-embedded larvae and the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) technique using the "DeadEnd colorimetric apoptosis detection system" (Promega). Peroxidase conjugated anti-digoxigenin secondary antibody and DAB as a substrate were used to obtain a specific brown reaction product. Panel A shows 3-day-old larvae, 5 h after an OA treatment. The DAB reaction product is localised in sporadic fatty cells in the larval haemocoel (➡). Magnification: 400 x. Panel B shows 5-day-old larvae, 21 h after an OA treatment. Sporadic columnar epithelial cells show brown DAB reaction products (➡). Magnification: 200 x.

Figure 6. Staining of formalin-fixed, paraffin-embedded larvae and the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) technique using "DeadEnd colorimetric apoptosis detection system" (Promega). Panel A shows 3-day-old larvae, 21 h after a formic acid (FA) treatment. The DAB reaction product localised to the cuticular (➡) and fat cells (▼) in the peripheral area of the larval body. Magnification: 100 x. Panel B shows 3-day-old larvae, 50 h after a formic acid (FA) treatment. The entire larval body shows localised DAB reaction products (*). Note DAB-negative midgut epithelial cells (▼). Magnification: 100 x.

4. DISCUSSION

Honeybee larvae treated with oxalic or formic acid undergo subclinical changes that are detected using immunohistochemical methods. ISCDDK applied to honeybee larvae, showed higher levels of cell death in comparison with the DeadEnd kit and higher induction of DNA-strand breaks after the OA or FA applications in the columnar but not in the regenerative cells. Epithelial cells show clear, nuclear localisation before they are sloughed off into the lumen (Alberts et al., 1994). ISCDDK was found to demonstrate DNA-fragmentation after both apoptotic and necrotic cell death (Matylevitch et al., 1998; Orita et al., 2000), while the Promega apoptosis kit demonstrated apoptosis only (Sperandio et al., 2000). It was also found that apoptosis and necrosis coexist and that an apoptotic process might become necrotic (Bell et al., 2001).

In our previous experiments where larvae were water-treated, 20% of the columnar cells were found positive after using ISCDDK (Gregorc and Bowen, 2000). Up to 5% apoptotic cell death in the larval midgut is indicative of a normal level of tissue turnover and has been previously established, using a histone-group protein and monitored free-acid phosphatase activity (Gregorc and Bowen, 1997, 1999). The small difference between cell death in untreated and OA-treated larvae using the DeadEnd kit clearly indicates

low levels of apoptosis. Some cytoplasmic staining seems to be indicative of DNA fragments released from the necrotically-changed epithelial cell nuclei 21 h after the OA or FA application. The percentage of epithelial cells labelled with digoxigenin using the ISCDDK increased to 70% in the 3-day-old larvae when applied to the FA-treated larvae. A difference was found in the proportion of columnar cells showing DNA-strand breaks when the ISCDDK was compared to the DeadEnd kit, where cell death remained steady at around 5%. The higher death rate of the midgut columnar cells is triggered by necrotic injury after an FA application. Necrosis, which is usually caused by a lethal accident or disease as opposed to being a programmed process, can still be detected by TUNEL as Orita et al. (1999), Pulkkanen et al. (2000) and Ortiz (2003) also found. The low-level cell death detected by the DeadEnd kit 50 h after the OA application seems to indicate normal cell turnover caused by apoptosis. The higher cell-death levels detected by the ISCDDK therefore indicate accidental cell death due to the OA application leading to necrosis.

An OA application to honeybee larvae affects the columnar cells of their midgut. It is clearly shown that 5-day-old larvae suffer greater damage in the midgut than 3-day-old larvae 50 h after an OA application. In the 3-day-old larvae, cell death was higher 21 h after the OA application than it was 50 h after the application. The lower cell death detected may have been a result of variations in the quantity of OA applied and consumed by the larvae and the sensitivity of young larvae to OA. Some larvae were removed from the comb cells after the first and second sampling of the bees, but remained alive and were still suitable for sampling. It has been demonstrated that an ISCDDK can show cell death in the skin tissue of a 2-week-old mouse (Sgonc and Gruber, 1998) and in honeybee larvae it appears to show apoptotic and necrotically-induced DNA changes (Gregorc and Bowen, 2000), indicating that apoptosis and accidental cell death were occurring simultaneously (Matylevitch et al*.,* 1998).

In OA-treated larvae, the increased cell death demonstrated with ISCDDK is accompanied with morphological characteristics of cytoplasm vacuolization, nuclear envelope expansion, and intercellular detachments. The increased cell death in the OA-treated larvae is due to necrosis as was ascertained by the ISCDDK, which shows both programmed and necrotic cell death (Orita et al., 1999; Gregorc and Bowen, 2000).

The study of cell death in honeybee larvae after the FA or OA applications has helped to better understand the possible adverse effects they may have and has resulted in establishing the different features of cell death, necrosis and apoptosis that could be detected using kits with different specificity (Orita et al., 1999; Sperandio et al., 2000). Quantification of cell death could be used in effectively monitoring the effects of organic acids on larval tissue, when applied to larvae in a honeybee colony. The ISCDDK and the DeadEnd kit served to detect the cellular responses of larval tissue suffering from "alternative" substances used against the bee parasite *Varroa destructor*. These methods are thus useful for evaluating the detrimental thresholds of larval tissue. Further clinical field tests to determine larval and/or adult bee death are needed to evaluate the full effects of substances used in bee colonies.

ACKNOWLEDGEMENTS

The financial support for the research project conducted by research group No. 502 of the Slovenian Ministry of Education, Science and Sport and CRP project V4-0759-02 and the financial support of The Royal Society (UK) and Slovenian Academy of Science are gratefully acknowledged.

Résumé – Mort cellulaire chez les larves de l'Abeille domestique traitées à l'acide oxalique ou à l'acide formique. L'acide oxalique (AO) et l'acide formique (AF) sont utilisés comme traitements alternatifs contre l'acarien *Varroa destructor*. Nous avons recherché si ces substances pouvaient induire une mort cellulaire et étudié sa répartition dans des larves d'*Apis mellifera* L. traitées à l'AO ou à l'AF. Une solution AO/saccharose a été préparée avec 6,5 g de dihydrate d'AO et 50 g de saccharose dans 100 mL d'eau désionisée. Chaque larve traitée a reçu environ 0,121 mg de solution d'AO. L'AF a été employé à la concentration de 85 % dans de l'eau désionisée et un évaporateur a été utilisé pour administrer le traitement.
Le 'kit de détection de mort cellulaire in situ AP' (ISCDDK, Roche) utilisé comprenait le mélange de réaction « TUNEL » composé de transférase avec un désoxynucléotide en position terminale (TdT). Tout enzyme TdT était détecté avec un « convertisseur AP » consistant en un anticorps antifluorescéine conjugué à la phosphatase alcaline. Le système de

détection colorimétrique de l'apoptose (= mort cellulaire programmée) DeadEnd (Promega) marque in situ l'ADN fragmenté des cellules apoptotiques. Un mélange de réaction TdT, ainsi qu'une solution de streptavidine marquée à la peroxydase de raifort, a servi à détecter les réactions, qui étaient ensuite visualisées à l'aide d'un substrat à base de DAB qui développe un arrière-plan foncé.
Cinq heures après le traitement, le produit de réaction était présent dans 25 % des noyaux des cellules columnaires de l'intestin moyen (Fig. 1) et 21 h après le traitement dans 76 % d'entre eux (Fig. 2). Au bout de 50 h le produit de réaction était localisé dans 18 % des cellules columnaires chez les larves âgées de 3 j et dans 81 % chez les larves de 5 j. Le produit de réaction a été trouvé de façon sporadique dans les cellules régénératives (Fig. 3a, b). Chez les larves traitées à l'AF un produit de réaction a été trouvé dans 70 % des noyaux des cellules columnaires mais pas dans les cellules régénératives. Il semble qu'il y ait une augmentation du degré de vacuolisation et de granulation du cytoplasme (Fig. 4). Le kit DeadEnd a montré la présence sporadique de cellules positives à la DAB chez des larves traitées à l'AO 5 h après le traitement (Fig. 5a). Des cellules positives à la DAB ont été trouvées dans environ 4 % des cellules épithéliales extrudées de l'intestin moyen 21 h après le traitement (Fig. 5b). Chez les larves traitées à l'AF, on a trouvé un produit à base de DAB dans les tissus périphériques, cuticulaires et sous-cuticulaires, et plus tard, dans les noyaux des cellules cuticulaires et sous-cuticulaires (Fig. 6a). Cinquante heures après le traitement il était étendu à l'ensemble des tissus larvaires (Fig. 6b). L'action de l'AF est visible dans la zone périphérique du corps des larves, là où les vapeurs de l'AF arrivent en contact direct avec la peau. Le niveau de mort cellulaire a augmenté avec le temps au cours de l'exposition. La faible différence dans la mort cellulaire entre les larves traitées à l'AO et les non traitées, mise en évidence par le kit DeadEnd, indique que une faible participation de l'apoptose. Chez les larves traitées à l'AO on observe une augmentation de la mort cellulaire due à la nécrose. L'ISCDDK et le kit DeadEnd permettent de détecter les réponses cellulaires des tisus larvaires atteints par les substances utilisées contre *V. destructor*.

***Apis mellifera* / mort cellulaire / acide oxalique / acide formique / méthode immunohistochimique**

Zusammenfassung – Zelltod bei Honigbienenlarven nach Behandlung mit Oxalsäure oder Ameisensäure. Larven von *Apis mellifera* L. wurden mit Oxalsäure (OA) oder Ameisensäure (FA) behandelt. Eine Oxalsäure/Zuckerlösung wurde aus 6,5 g OA Dihydrat und 50 g Zucker in 100 mL deionisiertem Wasser hergestellt. Jede behandelte Larve erhielt ungefähr 0,121 mg der OA Lösung. FA kam in einer 85 %igen Konzentration in deionisiertem Wasser unter Verwendung eines Verdunsters zur Anwendung.
Der angewendete 'In situ cell death detection kit, AP' (ISCDDK, Roche) beinhaltete die aus terminaler Deoxynucleotidyltransferase (TdT) bestehende "TUNEL" Reaktionsmischung. Jedes TdT Enzym wurde mit einem 'converter-AP' bestimmt, das aus einem mit Alkalinphosphatase konjugierten Anti-Fluorescein Antikörper besteht. Das "DeadEnd" colorimetric apoptosis detection system (Promega) kennzeichnet die fragmentierte DNA apoptotischer Zellen in situ. Eine TdT Reaktionsmischung zusammen mit einer mit Meerettisch-Peroxidase gelabelten Streptavidinlösung diente der Detektierung der Reaktionen, die dann mit einem DAB Substrat sichtbar gemacht wurden welches einen braunen Hintergrund entwickelt.
Fünf Stunden nach der OA Behandlung wurde in 25 % der Zellkerne der Mitteldarm-Säulenzellen Reaktionsprodukt gefunden (Abb. 1). 24 Stunden nach der OA-Behandlung stieg dieser Anteil auf 76 % (Abb. 2), nach fünfzig Stunden war das Reaktionsprodukt in 18 % der Säulenzellen von 3 Tage alten Larven vorhanden, bei 5 Tage alten Larven war es im Mittel auf 81 % angestiegen. Das Reaktionsprodukt fand sich sporadisch in regenerativen Zellen (Abb. 3a, b). Bei den FA-behandelten Larven wurde ein Reaktionsprodukt in 70 % der Kerne der Säulenzellen gefunden, nicht aber in den regenerativen Zellen. Anscheinend gab es einen Anstieg im Grad der Vakuolisierung und Granulierung des Zytoplasmas (Abb. 4). Das DeadEnd apoptosis detection kit zeigte 5 Stunden nach der Behandlung sporadische DAB-positive Zellen bei den OA-behandelten Larven, diese befanden sich überwiegend im larvalen Körperfett (Abb. 5a). Bei ungefähr 4 % der larvalen ausgezogenen Mitteldarm-Epithelzellen wurden 21 Stunden nach der OA-Anwendung DAB-positive Zellen gefunden (Abb. 5b). Bei den FA-behandelten Larven wurde das DAB Reaktionsprodukt in den peripheren, kutikulären und subkutikulären Geweben, später auch in den kutikulären und subkutikulären Zellkernen gefunden (Abb. 6a) und hatte sich 50 h nach der FA-Behandlung weiter ausgedehnt (Abb. 6b). Der Einfluss von FA kann im peripheren Bereich der Larven gesehen werden, in denen die FA Dünste in direkten Kontakt mit der Larvenhaut kommen. Der Grad des Zelltods nimmt im Laufe der Zeit zu, in der die Larven der Behandlung ausgesetzt sind. Der geringfügige Unterschied im Zelltod zwischen unbehandelten und OA-behandelten Larven bei Verwendung des DeadEnd kits deutet auf eine nur geringe Beteiligung von Apoptose. Bei den OA-behandelten Larven tritt eine Zunahme des Zelltods auf, die auf Nekrose zurückzuführen ist. Die Studie zeigt, dass das ISCDDK und das DeadEnd kit eingesetzt werden können, um die zelluläre Reaktion des Larvengewebes auf die gegen den Bienenparasiten *Varroa destructor* eingesetzten Substanzen aufzuklären.

***Apis mellifera* / Zelltod / Oxalsäure / Ameisensäure / immunohistochemische Methode**

REFERENCES

Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J.D. (1994) Differentiated cells and the maintenance of tissue, in: Molecular biology of the cell, 3rd ed., Garland Publishing Inc, New York & London, pp. 1139–1195.

Arends M.J., Wyllie A.H. (1991) Apoptosis: mechanisms and roles in pathology, Int. Rev. Exp. Pathol. 32, 223–254.

Bell H.S., Whittle I.R., Walker M., Leaver H.A., Wharton S.B. (2001) The development of necrosis and apoptosis in glioma: experimental findings using spheroid culture systems, Neuropathol. Appl. Neurobiol. 27, 291–304.

Bowen I.D., Mullarkey K., Morgan S.M. (1996) Programmed Cell Death during Metamorphosis in the Blow-Fly *Calliphora vomitoria*, Microsc. Res. Tech. (MRT) 34, 202–217.

Ellis R.E., Yuan J.Y., Horvitz H.R. (1991) Mechanisms and functions of cell death, Annu. Rev. Cell. Biol. 7, 663–698.

Grasl-Kraupp B., Ruttkay-Nedecky B., Koudelka H., Bukowska K., Bursch W., Schulte-Hermann R. (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: A cautionary note, Hepatology 21, 1465–1468.

Gregorc A., Bowen I.D. (1997) Programmed cell death in the honeybee (*Apis mellifera* L.*)* larvae midgut, Cell Biol. Int. 21, 151–158.

Gregorc A., Bowen I.D. (1998) Histopathological and histochemical changes in honeybee larvae (*Apis mellifera* L.) after infection with *Bacillus larvae*, the causative agent of American foulbrood disease, Cell Biol. Int. 22, 137–144.

Gregorc A., Bowen I.D. (1999) Heat-shock and histone group protein expression in honey-bee larvae infected with *Paenibacillus larvae,* Cell Biol. Int. 23, 211–218.

Gregorc A., Bowen I.D. (2000) The histochemical characterisation of cell death in honeybee larvae midgut after treatment with *Paenibacillus larvae*, Amitraz and Oxytetracycline, Cell Biol. Int. 24, 319–324.

Levy R., Benchaib M., Cordonier H., Souchier C., Guerin J.F. (1998) Annexin V labelling and terminal transferase-mediated DNA end labelling (TUNEL) assay in human arrested embryos, Mol. Hum. Reprod. 4, 775–783.

Matylevitch N.P., Schuschereba S.T., Mata J.R., Gilligan G.R., Lawlor D.F., Goodwin C.W., Bowman P.D. (1998) Apoptosis and accidental cell death in cultured human keratinocytes after thermal injury, Am. J. Pathol. 153, 567–577.

Moraes R.L.M.S., Bowen I.D. (2000) Modes of cell death in the hypopharyngeal gland of the honey bee (*Apis mellifera* L.), Cell Biol. Int. 24, 734–737.

Orita Y., Nishizaki K., Sasaki J., Kanda S., Kimura N., Nomiya S., Yuen K., Masuda Y. (1999) Does TUNEL staining during peri- and post-natal development of the mouse inner ear indicate apoptosis? Acta Otolaryngol. Suppl. 540, 6–22.

Ortiz A. (2003) Staining for cell death, The Lancet 361, 1748.

Papaefthimiou C., Pavlidou V., Gregorc A., Theophilidis G. (2002) The action of 2,4-dichlorophenoxyacetic acid on the isolated heart of insect and amphibia, Environ. Toxicol. Pharmacol. 11, 127–140.

Pulkkanen K.J., Laukkanen M.O., Naarala J., Yla-Herttuala S. (2000) False-positive apoptosis signal in mouse kidney and liver detected with TUNEL assay, Apoptosis, 5, 33–329.

Sgonc R., Gruber J. (1998). Apoptosis detection: an overview, Exp. Gerontol. 33, 525–533.

Short B.G., Zimmerman D.M., Schwartz L.W. (1997) Automated double labeling of proliferation and apoptosis in glutathione S-transferase-positive hepatocytes in rats, J. Histochem Cytochem. 45, 305–1299.

Sperandio S., de Belle I., Bredesen D.E. (2000) An alternative, nonapoptotic form of programmed cell death, Proc. Natl. Acad. Sci. (USA) 97, 14376–14381.

Trump B., Berezesky I. (1998) The reactions of cells to lethal injury – oncosis and necrosis, in: Lockshin R.A., Zakeri Z., Tilly J.L. (Eds.), When cells die, New York, Wiley-Liss., pp. 209–242.