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### ► **To cite this version:**

Jan Magdalan, Alina Ostrowska, Aleksandra Piotrowska, Agnieszka Gomulkiewicz, Marzena Podhorska-Okolów, et al.. Benzylpenicillin, acetylcysteine and silibinin as antidotes in human hepatocytes intoxicated with  $\alpha$ -amanitin. *Experimental and Toxicologic Pathology*, 2010, 62 (4), pp.367. 10.1016/j.etp.2009.05.003 . hal-00598190

**HAL Id: hal-00598190**

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## Author's Accepted Manuscript

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PII: S0940-2993(09)00181-X  
DOI: doi:10.1016/j.etp.2009.05.003  
Reference: ETP 50364

To appear in: *Experimental and Toxicologic Pathology*

Received date: 25 February 2009  
Accepted date: 17 May 2009

Cite this article as: Jan Magdalan, Alina Ostrowska, Aleksandra Piotrowska, Agnieszka Gomułkiewicz, Marzena Podhorska-Okołów, Dariusz Patrzalek, Adam Szelał and Piotr Dziegiel, Benzylpenicillin, acetylcysteine and silibinin as antidotes in human hepatocytes intoxicated with  $\alpha$ -amanitin, *Experimental and Toxicologic Pathology*, doi:10.1016/j.etp.2009.05.003

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**Benzylopenicillin, Acetylcysteine and Silibinin as Antidotes in Human Hepatocytes  
Intoxicated with  $\alpha$ -Amanitin**

Jan Magdalan<sup>a,\*</sup>, Alina Ostrowska<sup>a</sup>, Aleksandra Piotrowska<sup>b</sup>, Agnieszka Gomulkiwicz<sup>b</sup>,  
Marzena Podhorska-Okołów<sup>b</sup>, Dariusz Patrzalek<sup>c</sup>, Adam Szelağ<sup>a</sup>, Piotr Dziegiel<sup>b,d</sup>

<sup>a</sup>Department of Pharmacology, Wrocław Medical University, Wrocław, Poland

<sup>b</sup>Department of Histology and Embryology, Wrocław Medical University, Wrocław, Poland

<sup>c</sup>Department of Vascular, General and Transplantation Surgery, Wrocław Medical University,  
Wrocław, Poland

<sup>d</sup>Department of Histology and Embryology, Poznań University of Medical Science, Poznań,  
Poland

\*Corresponding author. Piotr Dziegiel M.D. Ph. D. Department of Histology and Embryology, Wrocław Medical University, Chalubinskiego 6a, PL 50-368 Wrocław, Poland.  
Tel/Fax: +48 71 784-00-82, E-mail: piotr@hist.am.wroc.pl

**Abstract**

Fatalities due to mushroom poisonings are increasing worldwide, with high mortality rate resulting from ingestion of amanitin-producing species. Intoxications caused by amanitin-containing mushrooms represent an unresolved problem in clinical toxicology since no specific and fully efficient antidote is available. The objective of this study was a comparative evaluation of benzylpenicillin (BPCN), acetylcysteine (ACC) and silibinin (SIL) as an antidotes in human hepatocytes intoxicated with  $\alpha$ -amanitin ( $\alpha$ -AMA).

All experiments were performed on cultured human hepatocytes. Cytotoxicity evaluation of cultured cells using MTT assay and measurement of lactate dehydrogenase (LDH) activity was performed at 12, 24 and 48 h of exposure to  $\alpha$ -AMA and/or antidotes.

The significant decline of cell viability and significant increase of LDH activity were observed in all experimental hepatocyte cultures after 12, 24, and 36 h exposure to  $\alpha$ -AMA at concentration 2  $\mu$ M. Exposure of the cells to  $\alpha$ -AMA resulted also in significant reduction of cell spreading and attachment. However, addition of tested antidotes to experimental cultures significantly stimulated cell proliferation and attachment. In cell cultures exposed simultaneously to  $\alpha$ -AMA and tested antidotes cytotoxic parameters (MTT and LDH) were not significantly different from control incidences. The cytoprotective effect of all antidotes was not dose-related, which reflects a high efficacy of all these substances. Administration of studied antidotes was not associated with any adverse effects in hepatocytes.

The administration of ACC, BPCN, or SIL to human hepatocyte cultures showed a similar strong protective effect against cell damage in  $\alpha$ -AMA toxicity.

**Key Words:**  $\alpha$ -amanitin, benzylpenicillin, acetylcysteine, silibinin, *Amanita phalloides*, intoxication, human hepatocytes

## Introduction

Amatoxin poisoning is caused by mushroom species belonging to the genera *Amanita*, *Galerina* and *Lepiota* with the majority of lethal mushroom exposures attributable to *Amanita phalloides* and its subspecies (*Amanita virosa*, *Amanita vernalis*) (Enjalbert et al., 2002; Schneider, 2001). Amatoxins are heat-stable octapeptides. A wide variety of amatoxins have been isolated, however,  $\alpha$ -amanitin ( $\alpha$ -AMA) appears to be the primary human toxin (Schneider, 2001). Treatment of poisonings caused by amatoxin-containing mushrooms includes gastrointestinal decontamination, supportive measures, antidotes, and if liver failure occurs, liver transplantation (Escudié et al., 2007; Schneider, 2001; Yildiz et al., 2008). Various substances widely used in the past to treat *Amanita phalloides* poisonings (steroids, cimetidine, thiocetic acid) are documented to be completely ineffective. Moreover, according to Enjalbert et al. (2002) the most often currently used antidote – benzylpenicillin (BPCN) shows poor clinical efficacy. Experimental and clinical data of the silibinin (SIL) and acetylcysteine (ACC) efficacy as an antidotes for amanitin toxicity are ambiguous (Enjalbert et al., 2002; Ganzert et al., 2008; Magdalan et al., in press; Saller et al., 2008; Schneider et al., 1992; Vogel et al., 1984; Tong et al., 2007). However, some clinical reports indicated that SIL or ACC appear to be more effective in mushroom poisoning therapy than BPCN (Enjalbert et al., 2002).

The review of therapeutic procedures reported on patients intoxicated with amatoxin-containing mushrooms demonstrates a wide variability in medical treatment and overall response rate (Enjalbert et al., 2002; Erguven et al., 2007; Ganzert et al., 2008; Giannini et al., 2007; Kotwica and Czerczak, 2007; Krenova et al., 2007). Thus, evaluation of different therapies of intoxications caused by amatoxin-producing mushrooms seems to be extremely difficult. A number of clinical factors need to be taken into account when evaluating an

effective treatment of mushroom poisoning, including but not limited to age and gender of the patient, health condition prior to intoxication, time from mushroom ingestion to onset of therapy, and clinical condition at the time of therapy initiation. Moreover, using specific treatment regimens including combined pharmacotherapy in one patient significantly complicates such evaluation (Enjalbert et al., 2002; Ganzert et al., 2008; Giannini et al., 2007). Therefore, primary human hepatocyte cultures are a good model for objective analysis of different antidotes without the necessity of taking into consideration all aforementioned clinical factors.

The objective of this study was a comparative evaluation of BPCN, ACC, and SIL efficacy in human hepatocytes intoxicated with  $\alpha$ -AMA.

## Materials and methods

### Hepatocyte isolation and culture

All experiments were performed after approval by the Local Ethics Commission at Wrocław Medical University, (Wrocław, Poland), license no. 481/2006. Media, supplements and reagents used for hepatocyte isolation and culture,  $\alpha$ -AMA and all tested antidotes were obtained from Sigma Poland Chem. Human liver was obtained through a network of organ procurement organization. At the time of referral the liver had been deemed unsuitable for organ transplantation. The liver was collected from brain-dead male donor and was protected from ischemic injury by flushing with ice-cold HTK solution immediately after vascular clamping. Hepatocytes were isolated from encapsulated left lateral segment of the liver as described previously (Ostrowska et al., 2000; Magdalan et al., 2009). After 4 h of initial incubation, the plating medium was substituted with defined culture medium (combination of

EBSS and Waymouth's 752/1, supplemented with 10% FBS). After the next 12 h incubation the medium was exchanged and primary hepatocyte cultures were maintained for 48 h with 1 daily dose of  $\alpha$ -AMA and/or antidotes (BPCN, ACC, SIL) at different final concentrations (Table 1).

ACC concentrations corresponded to its plasma levels obtained after the recommended dosage during the treatment in paracetamol toxicity (Prescott et al., 1989). BPCN concentrations corresponded to its plasma levels obtained after the dosage recommended in the therapy of the toadstool death cap poisoning, i.e. 300.000 – 1.000.000 U/kg/day (Heilmeyer et al., 1969; Schneider, 2001). SIL was used at concentrations corresponding to its therapeutic plasma levels (Kim et al., 2003; Lorenz et al., 1984).

Cytotoxicity evaluation of cultured cells was performed at 12, 24 and 36 h of exposure to  $\alpha$ -AMA and/or antidotes.

#### Analytical Methods

Retained functional integrity and viability of cultured hepatocytes was assessed using the MTT assay. Reduction of a yellow salt MTT by mitochondrial dehydrogenases in viable cells to a purple formazan precipitate was determined by measuring the absorbance at 570 nm on a plate reader (Elx 800 Universal Microplate Reader, Bio-Tek Instruments, USA). Lactate dehydrogenase (LDH) activity in hepatocyte culture supernatants was quantified spectrophotometrically by using dimension analyzer (Panda, DADE Behring RxL Max).

## Statistical analysis

Differences between values (MTT, LDH activity) were analyzed by one-way ANOVA with Tukey test using the Statistica 7.1 software (Stat Soft, Poland), and  $p < 0.05$  was considered statistically significant. Relationships between particular groups of the final data were evaluated by Spearman's correlation coefficient ( $r$ ).

## Results

The significant decline of cell viability and increase of LDH activity were observed in all experimental hepatocyte cultures after 12, 24, and 36 h exposure to  $\alpha$ -AMA at concentration 2  $\mu$ M (groups AMA). Following 12, 24, and 36 h evaluated cytotoxic parameters were identical in experimental cultures exposed to antidotes only (a, A, p, P, s, S) and in controls. In cell cultures exposed simultaneously to  $\alpha$ -AMA and tested antidotes (AMA-a, AMA-A, AMA-p, AMA-P, AMA-s, AMA-S) cytotoxic parameters determined (MTT, LDH) were not significantly different from control incidences (Figures 1-6).

There was a significant positive correlation between hepatocyte viabilities in cultures exposed simultaneously to  $\alpha$ -AMA and all tested antidotes (Table 2).

Exposure of cells to  $\alpha$ -AMA at concentration 2  $\mu$ M for 24 h resulted in significant reduction of cell viability and attachment. Addition of BPCN, SIL or ACC to experimental cultures significantly stimulated cell proliferation, spreading and attachment (Figure 7).



## Discussion

High mortality rate in intoxications caused by amatoxins-containing mushroom is principally a result of the acute liver failure following significant hepatocyte damage due to hepatocellular uptake of amanitins, the main amatoxins. Amanitins are absorbed through the intestinal mucosa and carried to the liver via the portal vein. Although in humans intestinal absorption of amanitins is very fast, the onset of clinical symptoms (watery diarrhea, abdominal pain, vomiting) occurs generally 10 - 12 h after mushroom ingestion. Therefore, patients with poisoning caused by amatoxin-containing mushroom typically seek medical care at the time when a large portion of toxins had been absorbed from the gastrointestinal tract and uptaken into hepatocytes (Jaeger et al., 1993; Schneider, 2001). The  $\alpha$ -AMA uptake by human hepatocytes is mediated by the OATP1B3, a subtype of organic anion-transporting polypeptide located in the plasma membrane (Letschert et al., 2006). Then,  $\alpha$ -AMA binds to the RPB1 subunit of RNA polymerase II, thereby blocking the synthesis of proteins including intracellular enzymes, leading to cell death (Nguyen et al., 1996; Rudd and Luse, 1996). The inhibitory or competitive OATP1B3 substrates may inhibit  $\alpha$ -AMA uptake by human hepatocytes (Letschert et al., 2006). Amanitins, including  $\alpha$ -AMA, remain in enterohepatic circulation for four days after mushroom ingestion (Jaeger et al., 1993; Schneider, 2001). Thus, administration of OATP1B3 substrates, including BPCN and SIL, can interrupt the enterohepatic circulation of amanitins and suppress their penetration into the cells, which can probably alleviate liver damage and improve treatment efficacy. Studies by Letscher et al. (2006) have indicated that OATP1B3 blockade, leading to suppression of amanitin influx into human hepatocytes, is the most probable main mechanism of BPCN and SIL action as antidotes in *Amanita phalloides* poisoning. SIL, the most active component of silymarin obtained from milk thistle, acts also as a scavenger of free radicals and exerts high anti-

inflammatory effect *in vivo* by inhibition of leukocyte migration into the inflamed site (De LaPuerta et al., 1996; Wellington and Jarvis, 2001). Moreover, SIL stimulates the hepatic regeneration through the stimulation of the protein synthesis. It binds to a specific binding site in the RNA polymerase I, stimulating the formation of ribosomes (Fraschini et al., 2002). The clinical evidence of a therapeutic effect of SIL in toxic liver diseases is scarce, however it can be concluded – concerning possible risk/probable benefits – that it is reasonable to employ silibinin or silymarin as a supportive therapy in poisonings with amanitin-containing mushroom (Ganzert et al., 2008; Saller et al., 2008). The similarity between clinical toxicity of  $\alpha$ -AMA and acetaminophen suggested ACC inclusion in the treatment of poisonings caused by amatoxins-producing mushrooms. Administration of ACC is beneficial in preventing hepatic injury in acetaminophen poisoning through stimulation of glutathione synthesis, enhancing transformation of toxic acetaminophen metabolite to non-toxic mercaptide conjugate (Anker, 2001). ACC acts also as a scavenger of free radicals formed in acetaminophen poisoning (Leuterburg et al., 1988). Kawaji et al. (1990) investigating amatoxins-producing mushroom toxicity in isolated rat hepatocytes showed that extract of *Amanita virosa* markedly decreased intracellular glutathione content. Moreover, according to some reports free radical formation might contribute to the severe amatoxin hepatotoxicity (Magdalan et al., 2008; Zheleva et al., 2007). Since there are no reports on standard dosage regimen in mushroom poisoning, we tested ACC in concentrations corresponding to its plasma levels after the recommended dosage during the treatment in acetaminophen toxicity.

In this study administration of BPCN, ACC or SIL to experimental human hepatocyte cultures showed a strong protective effect against cell damage in amanitin toxicity. Moreover, the significant positive correlation between hepatocyte viabilities in cultures exposed simultaneously to  $\alpha$ -AMA and all tested antidotes indicates that all tested antidotes (ACC, BPCN and SIL) showed a similar strong protective effect in amanitin toxicity. The

cytoprotective effect of all antidotes was not dose-related, which reflects a high efficacy of all these substances. Notably, this remarkable level of efficacy was demonstrated in hepatocyte cultures treated with both 10  $\mu\text{M}$  and 100  $\mu\text{M}$  of SIL. It can be considered especially important from clinical point of view, since in some countries the intravenous formulations of SIL are not registered and after oral dosage of special formulation of silymarin (Liverman's capsules), the maximum plasma concentration of SIL can reach 6.04  $\mu\text{g/ml}$ , i.e. 12  $\mu\text{M}$ . Whereas, maximum plasma concentration of SIL after administration of the conventional oral formulation of silymarin is low (1.13 – 1.33  $\mu\text{g/ml}$ ). Also oral bioavailability of pure SIL is very low due to its poor water solubility (Kim et al., 2003).

Freshly isolated hepatocytes showed rapid spreading and attachment to collagen-coated plates within 12 h after plating, and formed confluent monolayer by 24 h. The optimal cell attachment was maintained in all control cultures. Exposure of the cells to  $\alpha\text{-AMA}$  resulted in significant reduction of cell viability and attachment. However, addition of tested antidotes to experimental cultures significantly stimulated cell proliferation and attachment (Figure 7). Moreover, administration of all tested antidotes was not associated with any cytotoxic effects in hepatocytes.

Notably, our previous study demonstrated no protective effect of the same type of antidotes used in cultured canine hepatocytes exposed to  $\alpha\text{-AMA}$  (Magdalan et al., in press). Completely different results of those two separate experiments are most likely caused by interspecies differences. In the present study the addition of tested antidotes to experimental cultures of primary human hepatocytes significantly stimulated cell proliferation and viability, indicating high cytoprotective efficacy of all these substances. Whereas in case of canine hepatocytes all antidotes (BPCN, ACC and SIL) tested against toxic  $\alpha\text{-AMA}$  were not effective; cell viability based on MTT reaction as well as extracellular LDH activity in dog hepatocyte cultures were comparable to those of group receiving  $\alpha\text{-AMA}$  only. Furthermore,

the higher dose of  $\alpha$ -AMA (10  $\mu$ M) administered to canine hepatocyte cultures could likely produce major, irreversible damage to the cells, thus eliminating protective effect of used antidotes.

In summary, it can be concluded that administration of ACC, BPCN, or SIL to experimental human hepatocyte cultures showed a similar strong protective effect in amanitin toxicity. This study has revealed that the efficacy of cheaper and easily available antidotes like ACC and BPCN was comparable to expensive and in some countries unavailable SIL. However, further investigations are necessary to confirm clinical evidence of a therapeutic effect of these substances in mushroom poisoning.

### **Acknowledgements**

This work is a part of a project granted by Polish Ministry of Science and Higher Education (grant no. N 401 2809 33).

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## FIGURE LEGENDS

Figure 1. MTT activity in control and experimental groups (AMA; a; A; AMA-a; AMA-A). The number of viable hepatocytes (one representative hepatocyte preparation) is proportional to the MTT reaction product, as determined by the optical density. Each value represents the mean  $\pm$  SD, n = 16, \* $p$  < 0.05 vs. Control.

Figure 2. MTT activity in control and experimental groups (AMA; p; P; AMA-p; AMA-P). The number of viable hepatocytes (one representative hepatocyte preparation) is proportional to the MTT reaction product, as determined by the optical density. Each value represents the mean  $\pm$  SD, n = 16, \* $p$  < 0.05 vs. Control.

Figure 3. MTT activity in control and experimental groups (AMA; s; S; AMA-s; AMA-S). The number of viable hepatocytes (one representative hepatocyte preparation) is proportional to the MTT reaction product, as determined by the optical density. Each value represents the mean  $\pm$  SD, n = 16, \* $p$  < 0.05 vs. Control.

Figure 4. Lactic dehydrogenase activity in culture medium of control and in culture medium of experimental groups (AMA; a; A; AMA-a; AMA-A). Each value represents the mean  $\pm$  SD, n = 8, \* $p$  < 0.05 vs. Control.

Figure 5. Lactic dehydrogenase activity in culture medium of control and in culture medium of experimental groups (AMA; p; P; AMA-p; AMA-P). Each value represents the mean  $\pm$  SD, n = 8, \* $p$  < 0.05 vs. Control.

Figure 6. Lactic dehydrogenase activity in culture medium of control and in culture medium of experimental groups (AMA; s; S; AMA-s; AMA-S). Each value represents the mean  $\pm$  SD, n = 8, \* $p$  < 0.05 vs. Control.

Figure 7. Representative contrast-phase micrograph of human hepatocytes plated on collagen-coated dishes (group AMA-P, time 24 h, Magnification X 100).

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Table 1. Final concentrations of  $\alpha$ -AMA, ACC, BPCN, and SIL administered to human hepatocyte cultures. The cells were treated with 1 daily dose of antidote for 48 h.

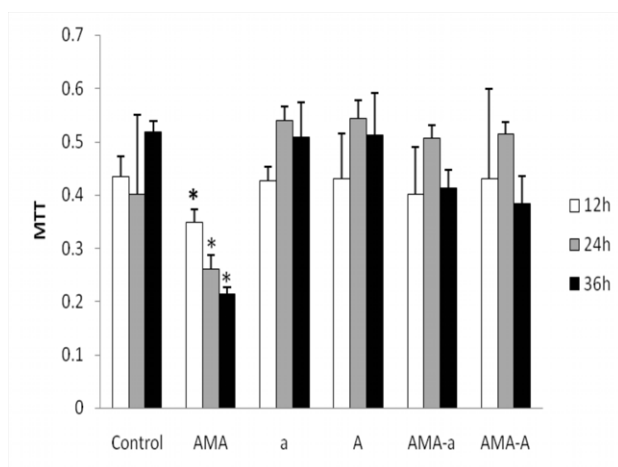
<b>Group</b>	<b><math>\alpha</math>-AMA (<math>\mu</math>M)</b>	<b>ACC (mM)</b>	<b>BPCN (mM)</b>	<b>SIL (<math>\mu</math>M)</b>
C				
AMA	2			
a		0.5		
A		1		
p			0.5	
P			1	
s				10
S				100
AMA-a	2	0.5		
AMA-A	2	1		
AMA-p	2		0.5	
AMA-P	2		1	
AMA-s	2			10
AMA-S	2			100

Table 2. The correlation between hepatocyte viabilities in cultures exposed simultaneously to  $\alpha$ -AMA and tested antidotes (r- Spearman's correlation coefficient).

Groups		r	p
AMA-A	AMA-P	0.87	<0.05
AMA-A	AMA-S	0.80	<0.05
AMA-P	AMA-S	0.93	<0.05

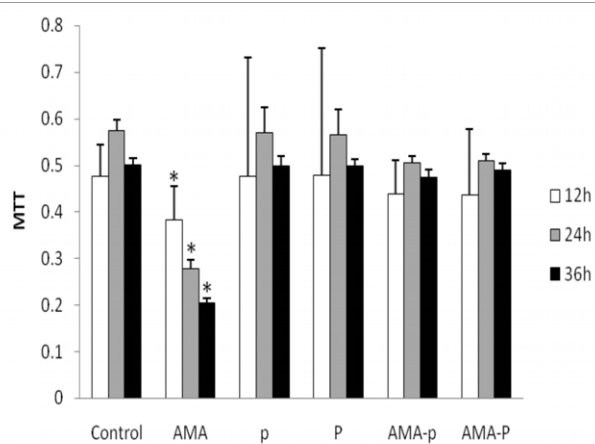
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Fig 1:



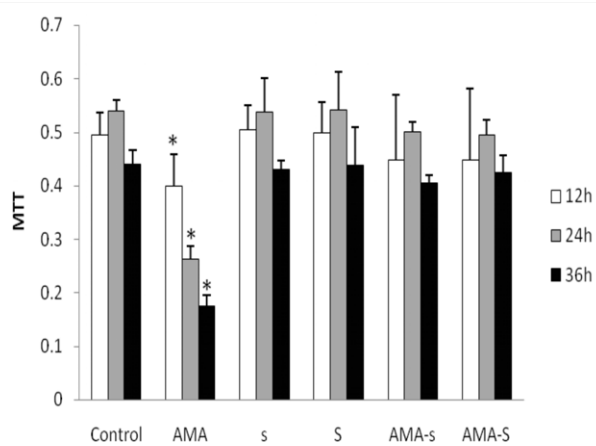
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Fig 2:



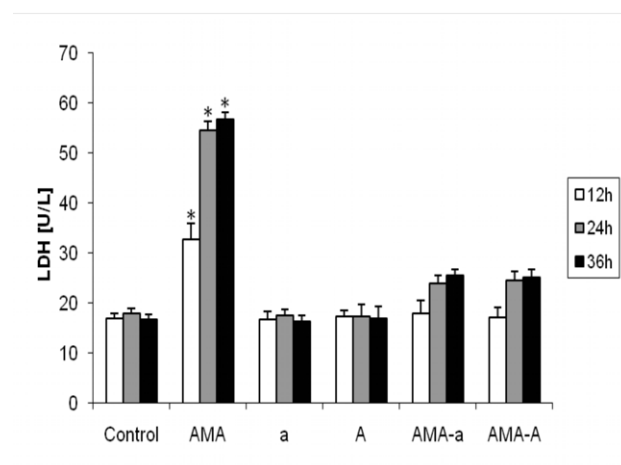
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Fig 3:



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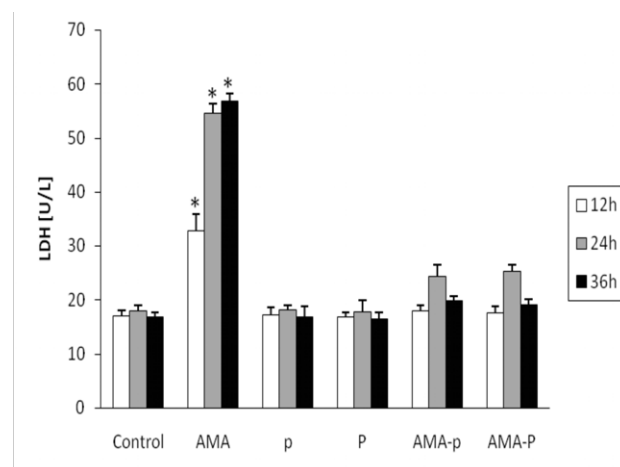
Fig 4:



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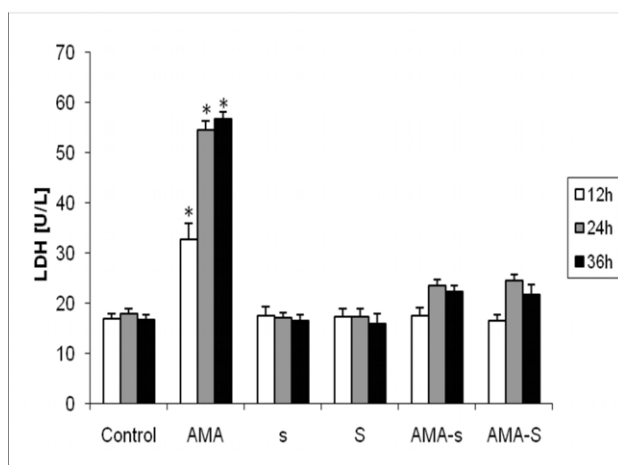


Fig 5:



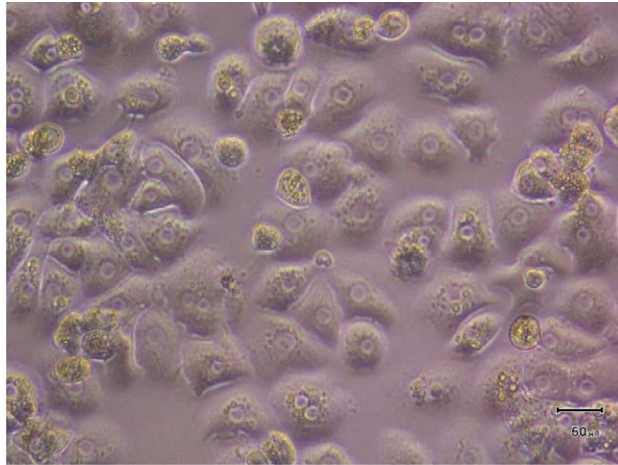
Accepted manuscript

Fig 6:



Accepted manuscript

Fig 7:



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