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In vitro toxicity of α-amanitin in human kidney cells and evaluation of protective effect of polymyxin B

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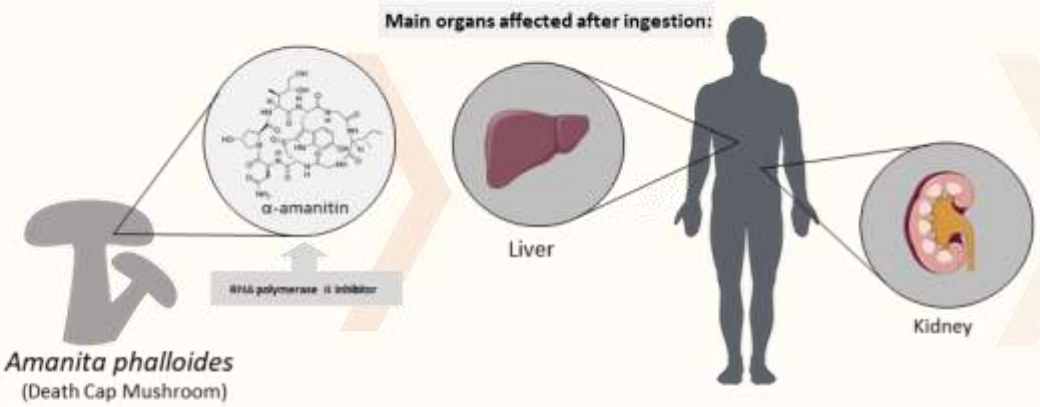
In vitro toxicity of α-amanitin in human kidney cells and evaluation of protective effect of polymyxin B

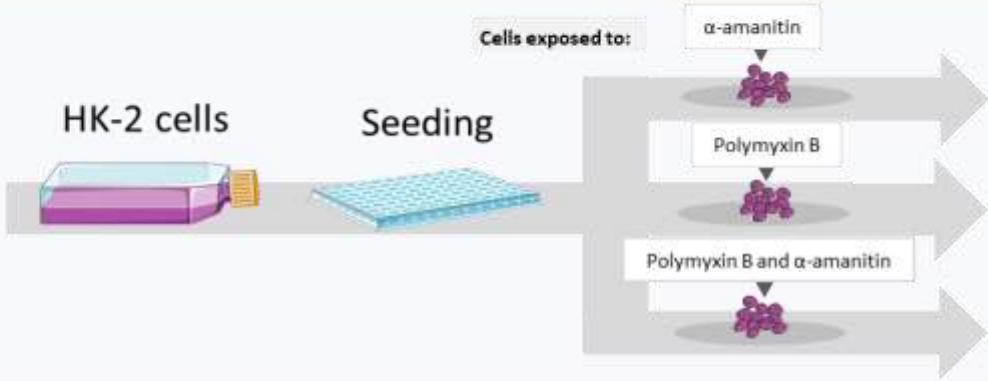
Background



Polymyxin B was shown to be an effective antidote *in vivo*, in Charles River mice

Does Polymyxin B confer protection against α-amanitin cytotoxicity in renal cells?

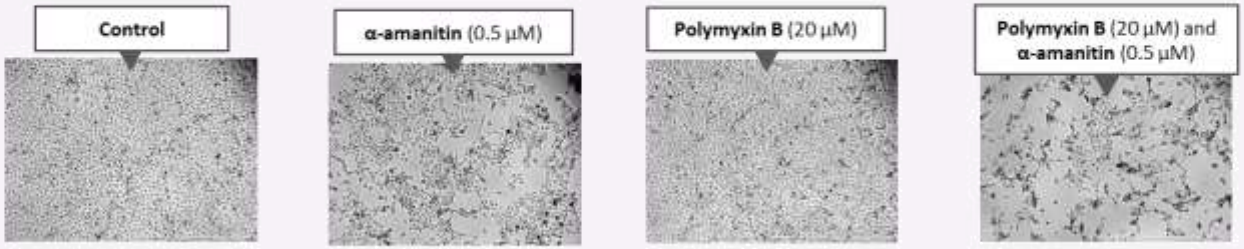
Methods



Cell Viability Assays

MTT reduction and NR uptake assays

Results



Abstract:

α-Amanitin intoxications have been associated with acute kidney injury and renal failure, besides its well-known hepatotoxic effects. Currently, no effective antidote against α-amanitin toxicity exists. Recent *in vivo* studies have shown that polymyxin B (PolB) decreases α-amanitin toxicity and that the associated renal damage is largely decreased by this antibiotic. This work aimed to characterize α-amanitin cytotoxicity in HK-2 cells and evaluate PolB's putative antidotal effectiveness in this in vitro system.

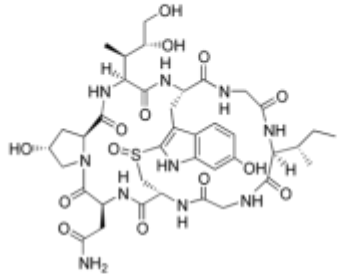
HK-2 cells were exposed to α-amanitin (0.01-10 µM) at different time-points and cytotoxicity evaluated by the MTT reduction and neutral red uptake assays. To assess PolB putative protective effects, two paradigms were used: (i) 30 min pre-incubation with PolB followed by 48h incubation with α-amanitin (0.5 and 1 µM) or (ii) PolB co-incubation with α-amanitin (5 and10 µM) for 2h followed by a 48h drug/toxin-free period.

α-Amanitin led to cytotoxicity effects on kidney cells at clinical relevant concentrations. The effectiveness of a previously described antidote, PolB, was not verified *in vitro,* which highlights the importance of further investigation on this antidotal strategy and its mechanisms.

Keywords: Amatoxin; Nephrotoxicity; Antidote; Poisoning

Introduction

Amanita Phalloides



Chemical structure of α-amanitin

Amanita Phalloides (also known as death cap mushroom) is **responsible for more than 90% of the fatalities caused by mushroom poisonings worldwide.**

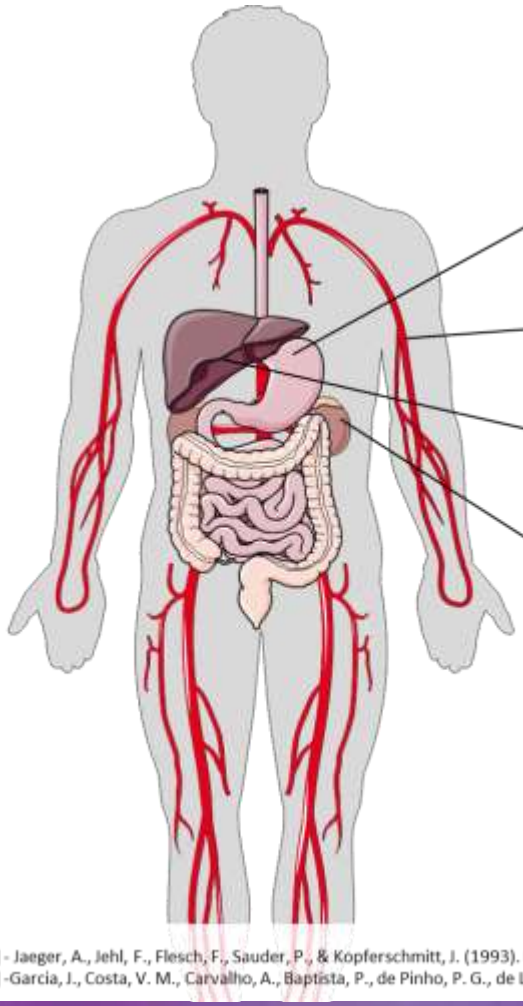
Amanita phalloides high lethality relies on powerful toxins such as α-amanitin.

α-Amanitin is a bicyclic octapeptide toxin belonging to the amatoxin family.

α-Amanitin is heat resistant, resistant to enzymatic and acidic degradation.

[1] - Ward, J., Kapadia, K., Brush, E., & Salhanick, S. D. (2013). Amatoxin poisoning: case reports and review of current therapies. *The Journal of emergency medicine*, *44*(1), 116-121.
[2] - Garcia, J., Costa, V. M., Carvalho, A. T., Silvestre, R., Duarte, J. A. et al. (2015). A breakthrough on Amanita phalloides poisoning: an effective antidotal effect by polymyxin B. *Archives of toxicology*, *89*(12), 2305-2323.
[3] - Pahl, A., Lutz, C., & Hechler, T. (2018). Amanitins and their development as a payload for antibody-drug conjugates. *Drug Discovery Today: Technologies*, *30*, 85-89.

Introduction



1. Amatoxins are readily absorved from the intestinal tract and can be detected as early as 90-120 min in urine following ingestion.
2. Amatoxins are present in plasma at low concentrations during a short period of 24-48h following ingestion.
3. Amatoxins are not greatly metabolized and are rapidly distributed to the liver and kidney.
4. α-Amanitin is mainly eliminated through kidneys, where a high concentration is detected.

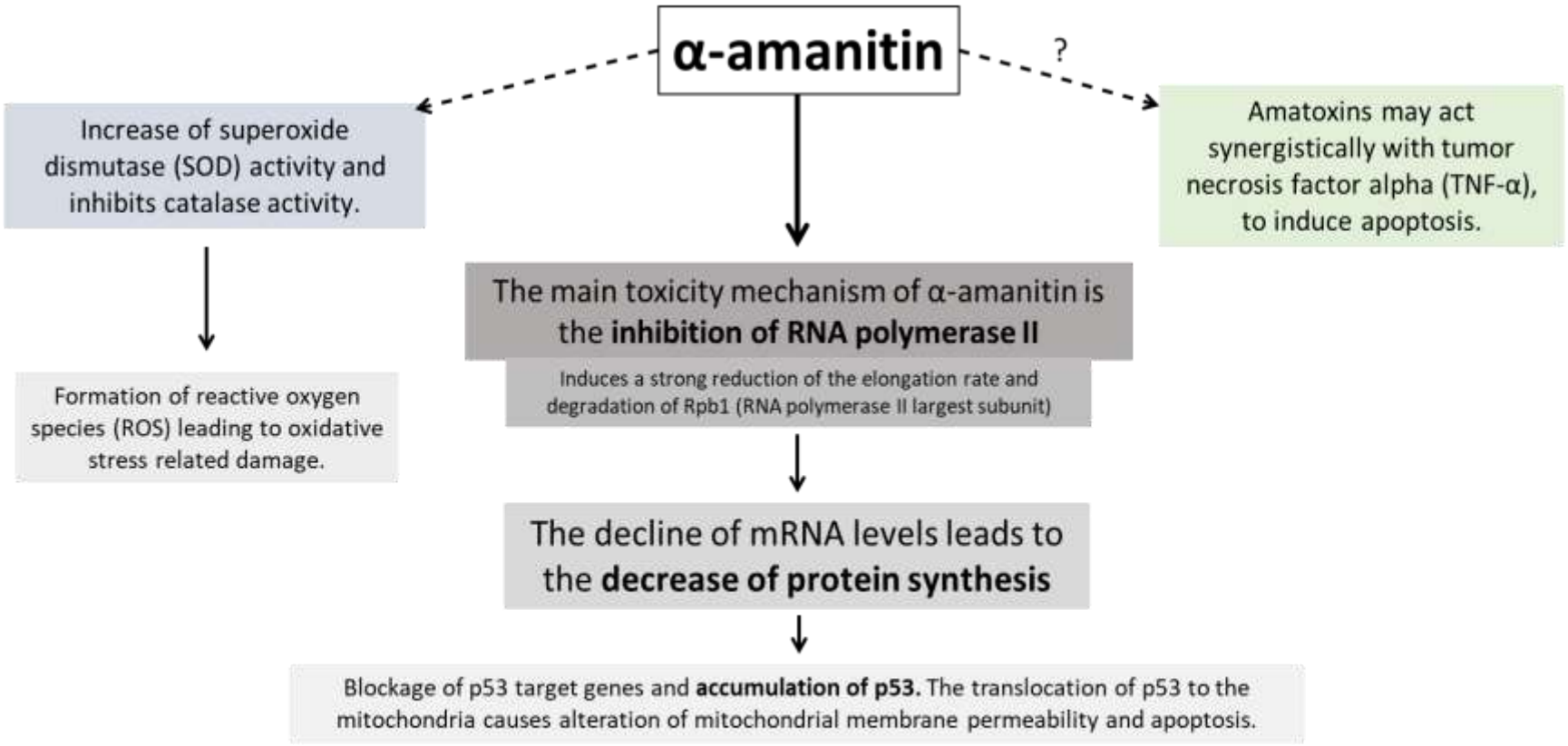
↓

Nefrotoxicity and renal failure

Currently, no effective antidote against α-amanitin toxicity is established.

[4] - Jaeger, A., Jehl, F., Flesch, F., Sauder, P., & Kopferschmitt, J. (1993). Kinetics of amatoxins in human poisoning: therapeutic implications. *Journal of Toxicology: Clinical Toxicology*, *31*(1), 63-80.
[5] -Garcia, J., Costa, V. M., Carvalho, A., Baptista, P., de Pinho, P. G., de Lourdes Bastos, M., & Carvalho, F. (2015). Amanita phalloides poisoning: Mechanisms of toxicity and treatment. *Food and chemical toxicology*, *86*, 41-55.

Introduction



[5] - Garcia, J., Costa, V. M., Carvalho, A., Baptista, P., de Pinho, P. G., de Lourdes Bastos, M., & Carvalho, F. (2015). Amanita phalloides poisoning: Mechanisms of toxicity and treatment. *Food and chemical toxicology*, *86*, 41-55.

Introduction

Arch Toxicol (2015) 89:2305–2323
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MOLECULAR TOXICOLOGY

A breakthrough on *Amanita phalloides* poisoning: an effective antidotal effect by polymyxin B

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Abstract *Amanita phalloides* is responsible for more than 90 % of mushroom-related fatalities, and no effective antidote is available. α-Amanitin, the main toxin of *A. phalloides*, inhibits RNA polymerase II (RNAP II), causing hepatic and kidney failure. *In silico* studies included docking and molecular dynamics simulation coupled to molecular mechanics with generalized Born and surface area method energy decomposition on RNAP II. They were performed with a clinical drug that shares chemical similarities to α-amanitin, polymyxin B. The results show that polymyxin B potentially binds to RNAP II in the same interface of α-amanitin, preventing the toxin from binding to RNAP II. In vivo, the inhibition of the mRNA transcripts elicited by α-amanitin was efficiently reverted by polymyxin B in the kidneys. Moreover, polymyxin B significantly decreased the hepatic and renal α-amanitin-induced injury as seen by the histology and hepatic aminotransferases plasma data. In the survival assay, all animals exposed to α-amanitin died within

Moreover, a single dose of polymyxin B administered concomitantly with α-amanitin was able to guarantee 100 % survival. Polymyxin B protects
tion leading to an effective preven
increasing survival in α-amanitin-
sent use of clinically relevant co
human-use-approved drug prompt
as an antidote for *A. phalloides* po

Keywords α-Amanitin · RNA p
B · Liver · Kidney

Introduction

The gathering and consumption of wild mushrooms has increased during recent years due to their delicate flavors and textures as well as their attributed high nutritional value

In vivo, the inhibition of the mRNA transcripts elicited by α-amanitin was efficiently reverted by polymyxin B in the kidneys. Moreover, polymyxin B significantly decreased the hepatic and renal α-amanitin-induced injury as seen by the histology and hepatic aminotransferases plasma data.

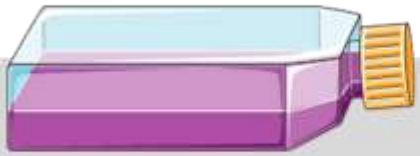
Moreover, a single dose of polymyxin B administered concomitantly with α-amanitin was able to guarantee 100 % survival. Polymyxin B protects RNAP II from inactivation leading to an effective prevention of organ damage and increasing survival in α-amanitin-treated animals.

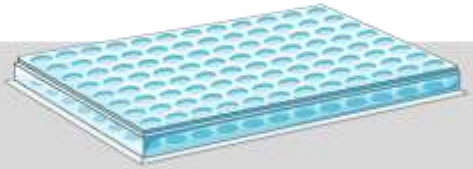
Introduction

Aims

This work aims to characterize α-amanitin cytotoxicity in renal HK-2 cells and evaluate the putative protective effects of polymyxin B.

Methods





Evaluation of α-amanitin cytotoxicity

Evaluation of Polymyxin B cytotoxicity

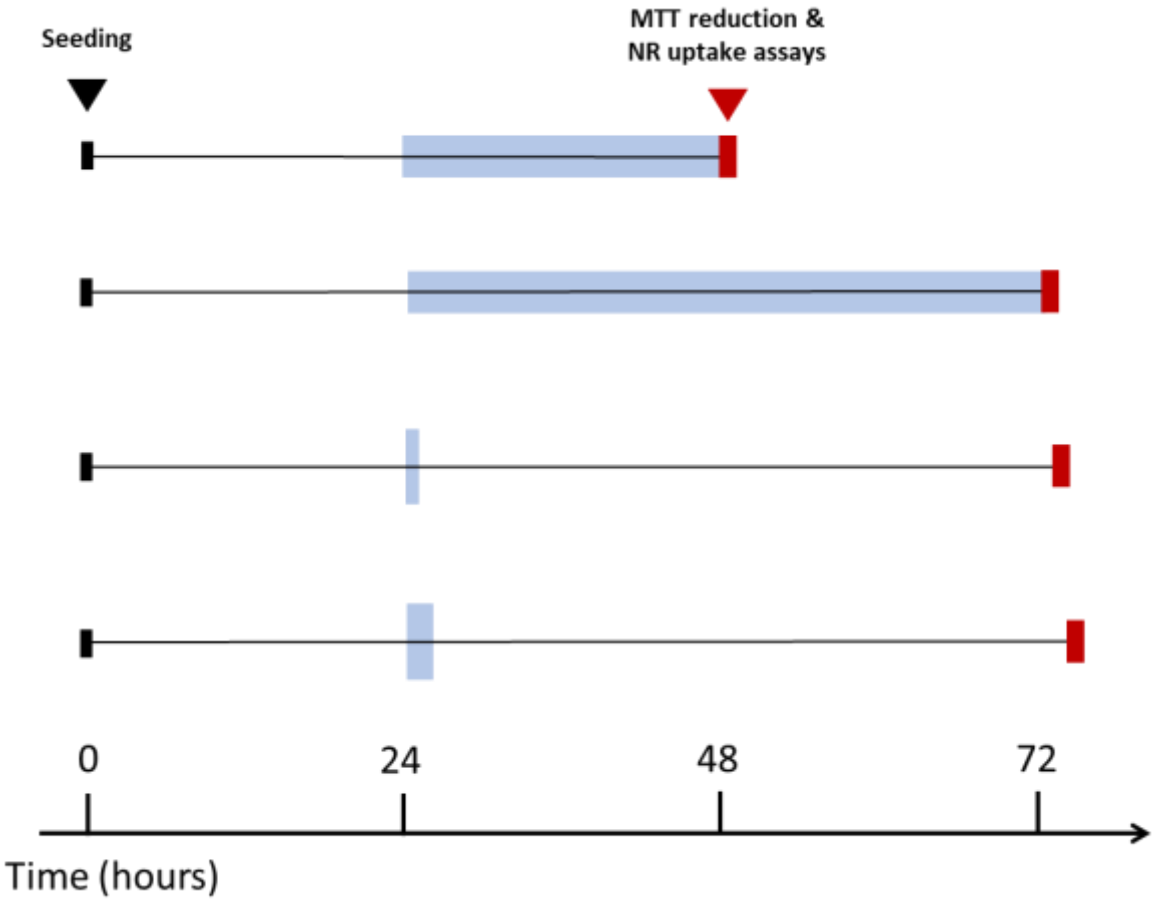
Protective effects of Polymyxin B against α-amanitin cytotoxicity

HK-2 cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin at 37ºC with 5% CO_2.

Cells were seeded in a density 15625 cells/cm² in 96 well-plates. All experiments were carried out between passage 8 and 15, 24h after trypsinization.

Cytotoxicity was evaluated by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide **(MTT) reduction** and neutral red **(NR) uptake assays**.

Cytotoxicity evaluation of α-amanitin



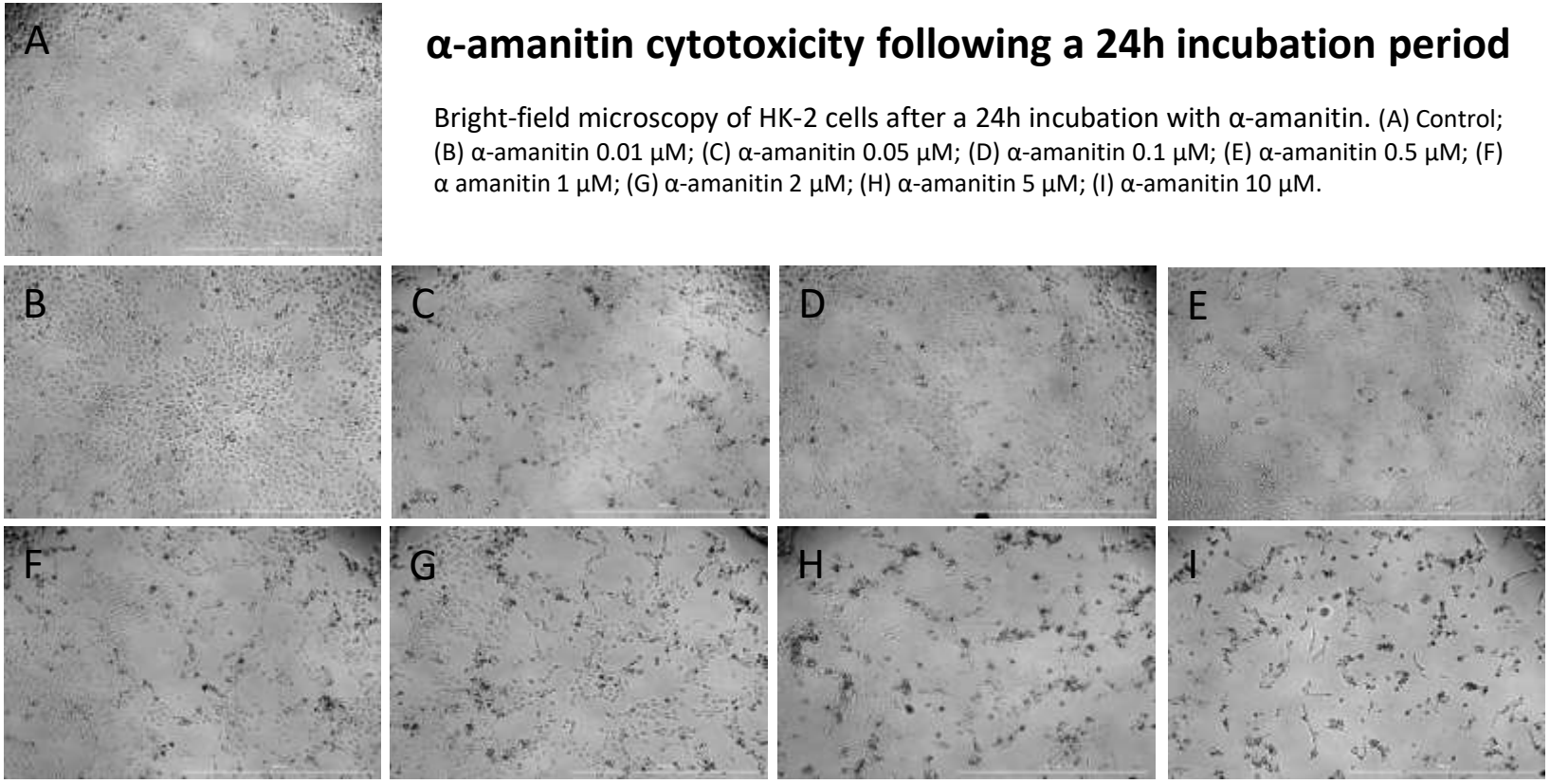
Incubation with α-amanitin (0.01 – 10 μM) for a **24-h period**

Incubation with α-amanitin (0.01 – 10 μM) for a **48-h period**

1-h incubation with α-amanitin (1-10 μM) followed by a 48-h α-amanitin-free period

2-h incubation with α-amanitin (1-10 μM) followed by a 48-h α-amanitin-free period

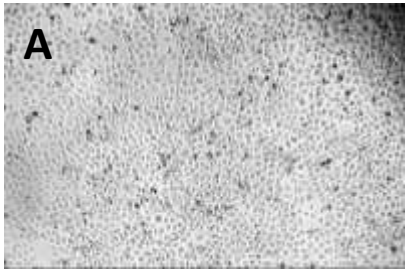
Results and discussion



α-amanitin cytotoxicity following a 24h incubation period

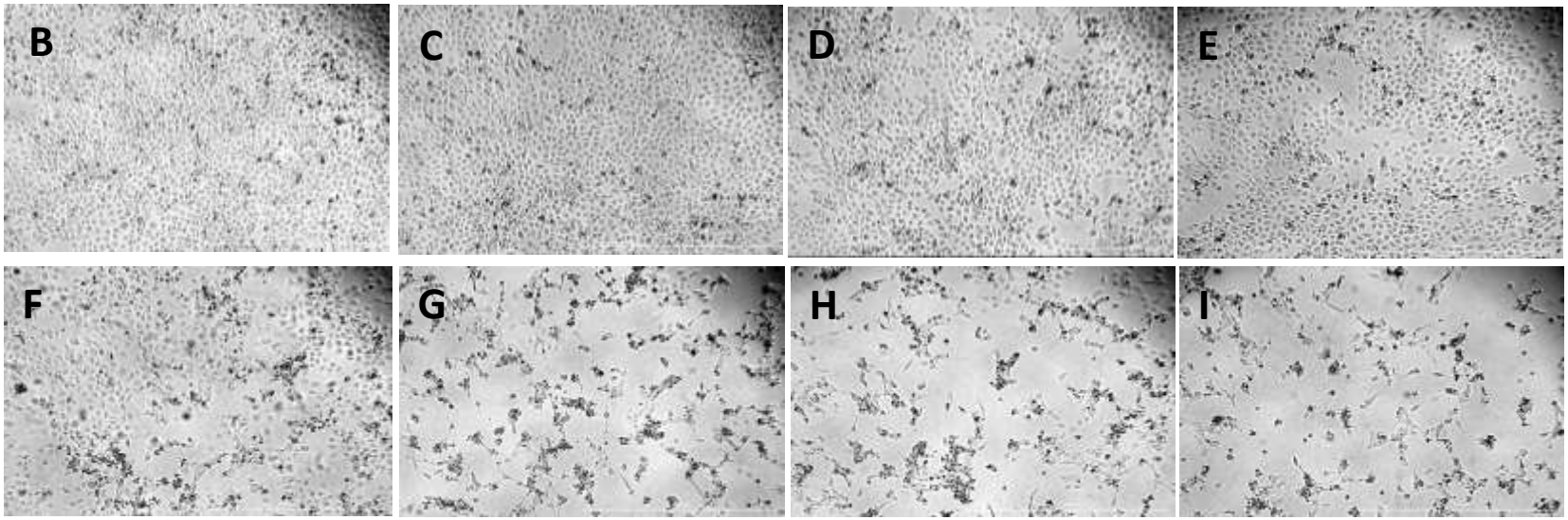
Bright-field microscopy of HK-2 cells after a 24h incubation with α-amanitin. (A) Control; (B) α-amanitin 0.01 µM; (C) α-amanitin 0.05 µM; (D) α-amanitin 0.1 µM; (E) α-amanitin 0.5 µM; (F) α amanitin 1 µM; (G) α-amanitin 2 µM; (H) α-amanitin 5 µM; (I) α-amanitin 10 µM.

Results and discussion



α-amanitin cytotoxicity following a 48h incubation period

Bright-field microscopy of HK-2 cells after a 48h incubation with α-amanitin. (A) Control; (B) α-amanitin 0.01 µM; (C) α-amanitin 0.05 µM; (D) α-amanitin 0.1 µM; (E) α-amanitin 0.5 µM; (F) α amanitin 1 µM; (G) α-amanitin 2 µM; (H) α-amanitin 5 µM; (I) α-amanitin10 µM.



Results and discussion

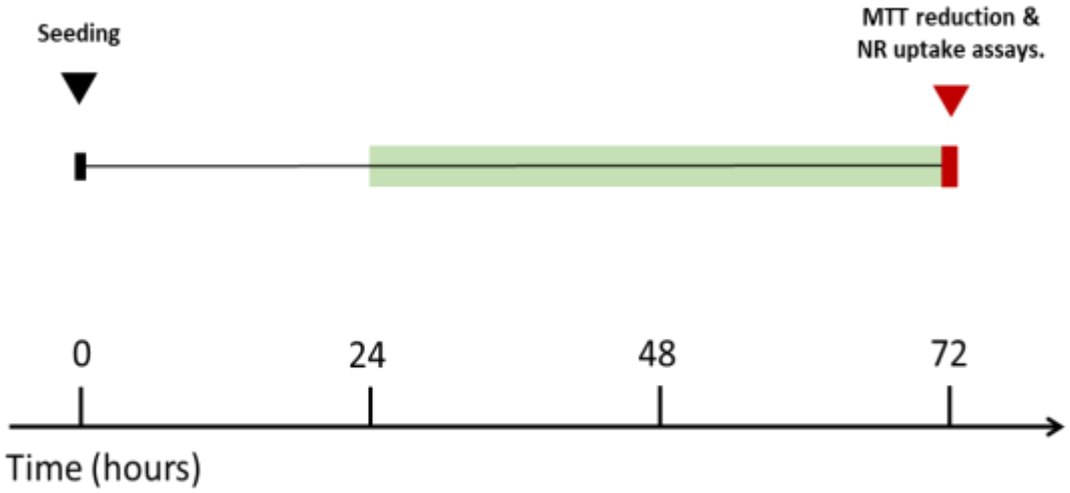
α-amanitin cytotoxicity after 1h incubation followed by a 48h α-amanitin-free period

	Cell viability assays	
[α-amanitin]	MTT reduction	NR uptake
1 µM	↓	=
2 µM	=	↓
5 µM	↓↓↓↓	↓↓↓↓
10 µM	↓↓↓↓	↓↓↓↓

α-amanitin cytotoxicity after 2h incubation followed by a 48h α-amanitin-free period

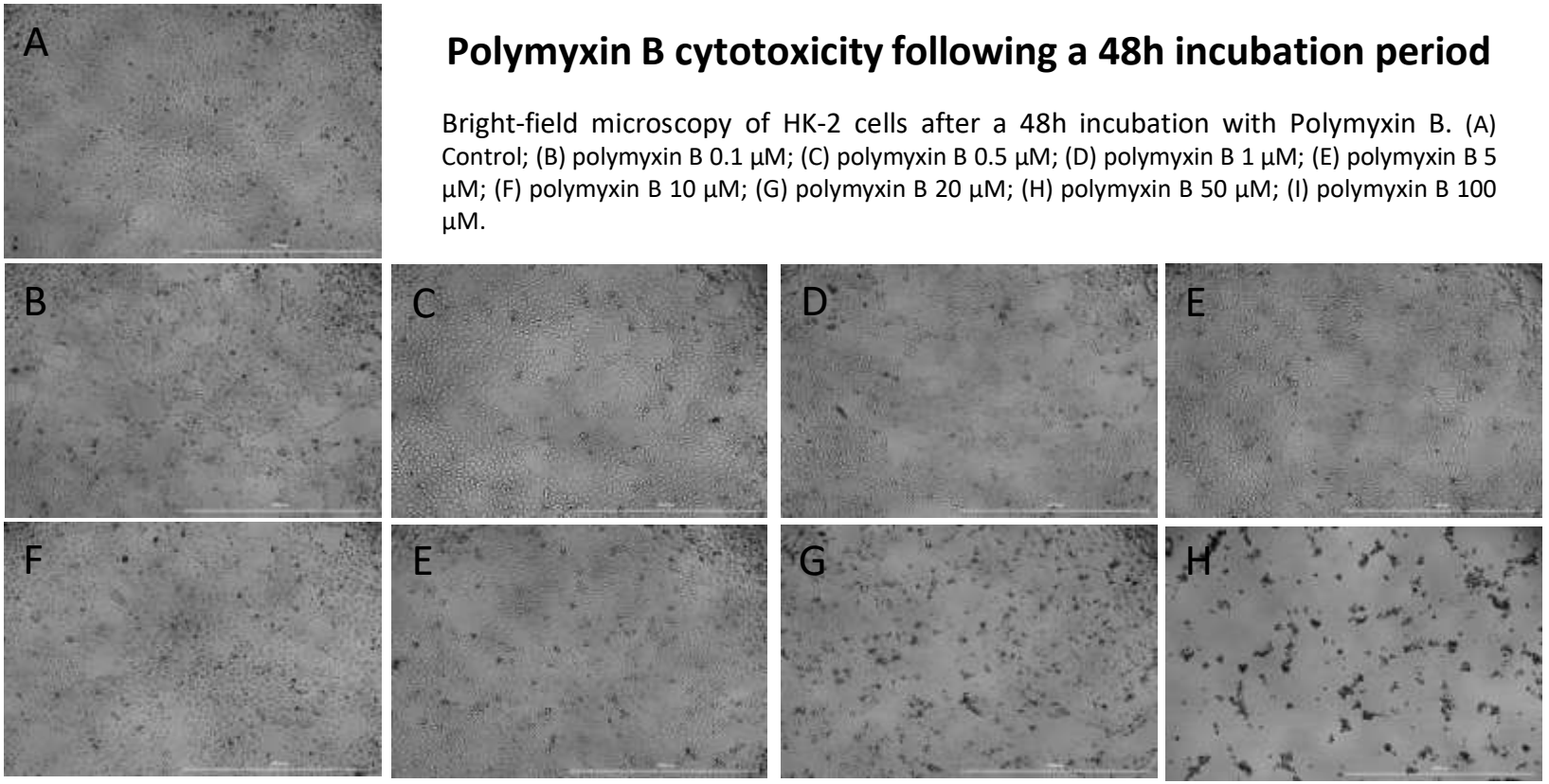
	Cell viability assays	
[α-amanitin]	MTT reduction	NR uptake
1 µM	↓↓	↓↓
2 µM	↓↓↓↓	=
5 µM	↓↓↓↓	↓↓↓↓
10 µM	↓↓↓↓	↓↓↓↓

Cytotoxicity evaluation of Polymyxin B at 48h



Incubation with **Polymyxin B** (0.1 – 100 µM**) for a 48-h period**

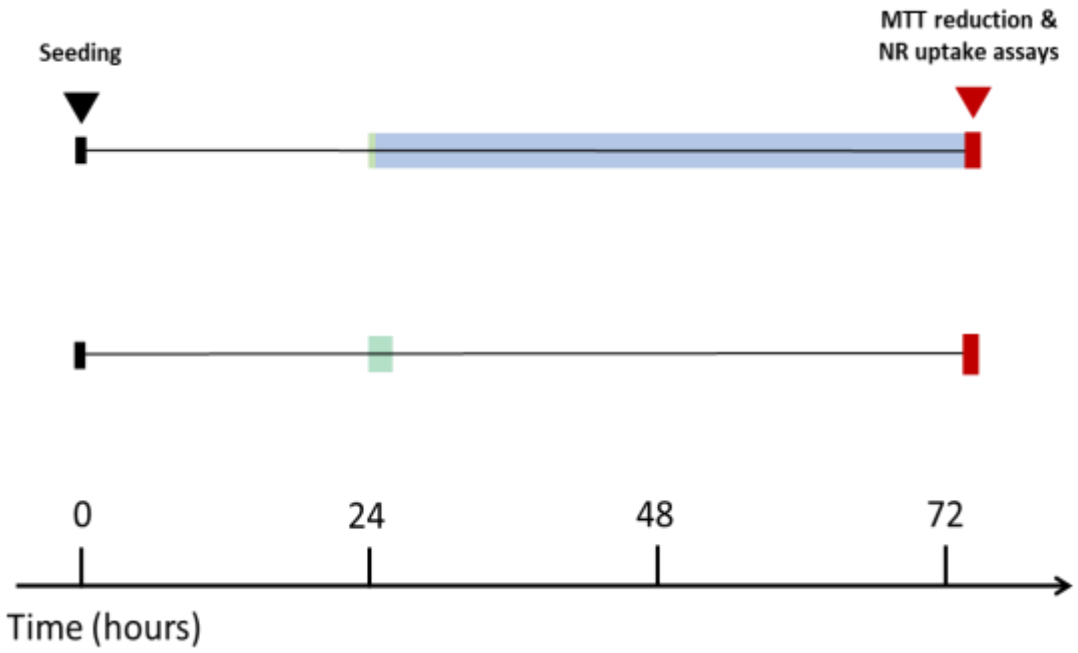
Results and discussion



Polymyxin B cytotoxicity following a 48h incubation period

Bright-field microscopy of HK-2 cells after a 48h incubation with Polymyxin B. (A) Control; (B) polymyxin B 0.1 µM; (C) polymyxin B 0.5 µM; (D) polymyxin B 1 µM; (E) polymyxin B 5 µM; (F) polymyxin B 10 µM; (G) polymyxin B 20 µM; (H) polymyxin B 50 µM; (I) polymyxin B 100 µM.

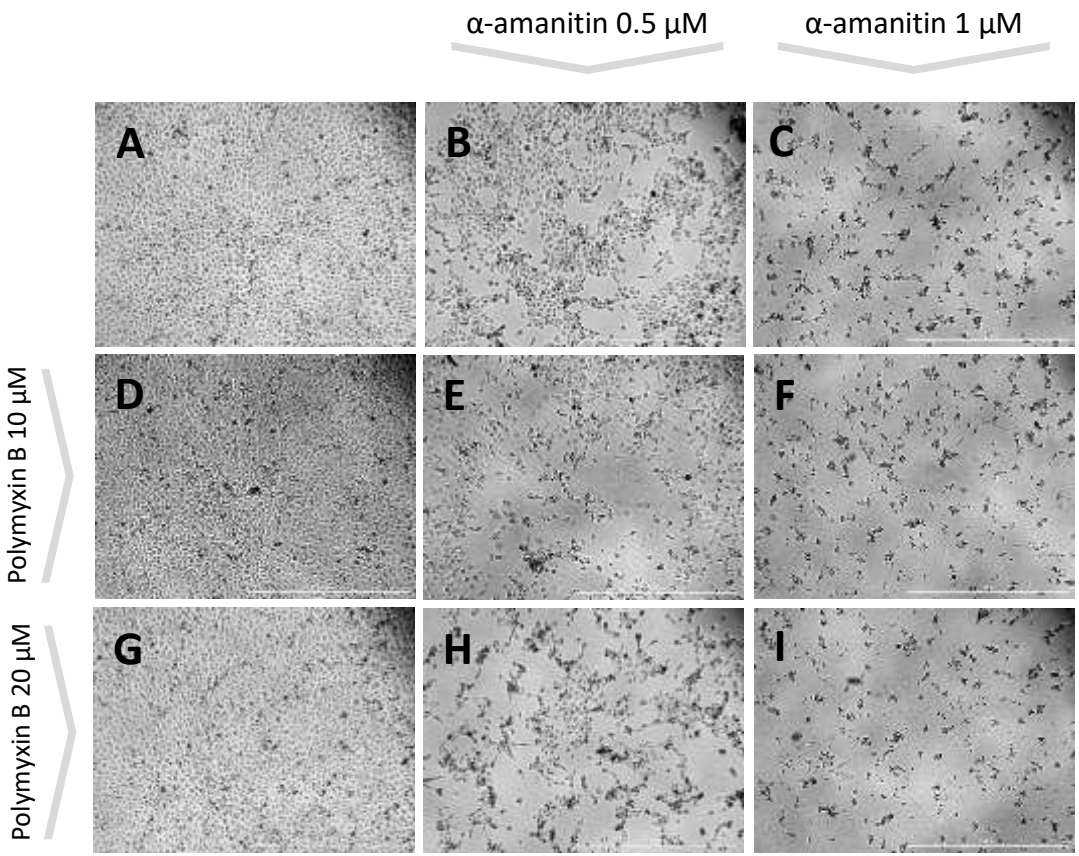
Putative effects of Polymyxin B against α-amanitin



30min pre-incubation with Polymyxin B (10 or 20 µM) followed by **48h incubation with α-amanitin** (0.5 or 1 µM)

Polymyxin B (5 or 10 µM **co-incubation with α-amanitin** (20 or 50 µM) **for 2h** followed by a **48h drug/toxin-free period**

Results and discussion

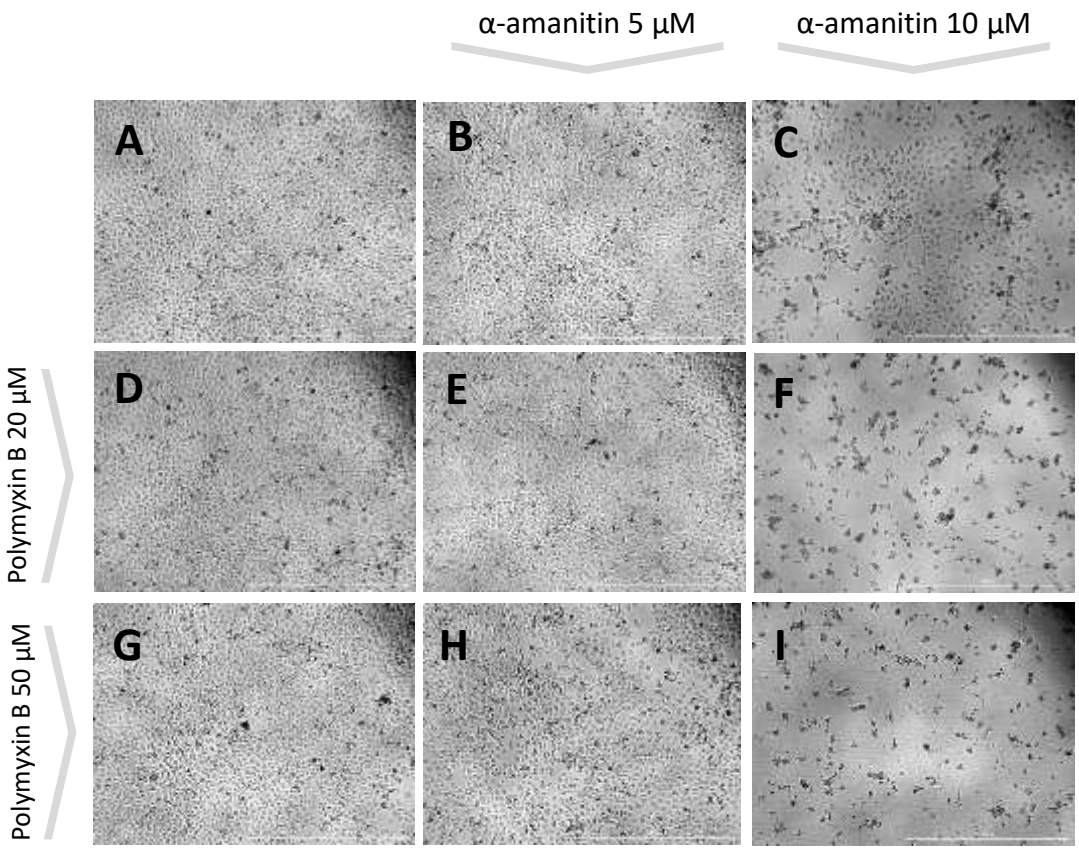


Protective effects of Polymyxin B against α-amanitin:

Bright-field microscopy of HK-2 cells after **30min pre-incubation with Polymyxin B followed by 48h incubation with α-amanitin.** (A) Control; (B) α-amanitin 0.5 µM (C) α-amanitin 1 µM; (D) Polymyxin B 10 µM; (E) α-amanitin 0.5 µM + Polymyxin B 10 µm; (F) α-amanitin 1 µM + Polymyxin B 10 µM; (G) Polymyxin B 20 µM; (H) α-amanitin 0.5 µM + Polymyxin B 20 µM; (I) α-amanitin 1 µM + Polymyxin B 20 µM.

No difference was observed between cells exposed to α-amanitin and Polymyxin B and cells exposed to α-amanitin alone.

Results and discussion



Protective effects of Polymyxin B against α-amanitin:

Bright-field microscopy of HK-2 cells after **Polymixin B co-incubation with α-amanitin for 2h followed by a 48h drug/toxin-free period.** (A) Control; (B) α-amanitin 5 µM (C) α-amanitin 10 µM; (D) Polymyxin B 20 µM; (E) α-amanitin 5 µM + Polymyxin B 20 µM; (F) α-amanitin 10 µM + Polymyxin B 20 µM; (G) Polymyxin B 50 µM; (H) α-amanitin 5 µM + Polymyxin B 50 µM; (I) α-amanitin 10 µM + Polymyxin B 50 µM.

No difference was observed between cells exposed to α-amanitin and Polymyxin B and cells exposed to α-amanitin alone.

Conclusions

- **The observed α-amanitin toxicity was time- and concentration-dependent;**
  - → α-Amanitin toxicity was observed within 24h at concentrations higher than 1 µM in the MTT reduction assay;
  - → After a 48h incubation, α-amanitin caused significant cytotoxicity above 0.5 µM.
- **Lower toxicity was observed in shorter incubation periods (1 or 2h)**
  - → a 5 times higher concentration was needed to obtain a similar effect to the 48h continuous incubation;
    - → α-amanitin uptake by HK-2 cells is slow.
- **Polymyxin B did not cause significant toxicity in concentrations bellow 100 µM after a 48h incubation period in the MTT reduction assay.**
- **Polymyxin B did not confer protection against α-amanitin cytotoxicity in all experimental paradigms tested.**

Acknowledgments

This work was supported by FEDER funds through the Operational Programme for Competitiveness Factors – COMPETE and by national funds by the FCT within the project PTDC-DTP-FTO-4973-2014 – POCI-01-0145-FEDER 016545. VMC acknowledges Fundação da Ciência e Tecnologia (FCT) for her grant (SFRH/BPD/110001/2015), that was funded by national funds through FCT – Fundação para a Ciência e a Tecnologia, I.P., under the Norma Transitória – DL57/2016/CP1334/CT0006.