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ORIGINAL ARTICLE

# Cytotoxicity of ketamine, xylazine and Hellabrunn mixture in liver-, heart- and kidney-derived cells from fallow deer

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## Abstract

**OBJECTIVES:** Chemical restraint of wild animals is practiced to accomplish intended procedures such as capture, clinical examination, collection of diagnostic samples, treatment and/or transport. Extra-label use of animal medicinal drugs is often necessary in wildlife because most approved therapeutics do not list wild species on the labelling. Here, we used cellular *in vitro* models, a cutting-edge tool of biomedical research, to examine cytotoxicity of anaesthetic agents in fallow deer and extrapolate these data for anaesthetic risks in wildlife.

**METHODS:** We examined the cytotoxic effects of ketamine, xylazine, and ketamine-xylazine, i.e. the Hellabrunn mixture, on liver-, heart- and kidney-derived cell cultures prepared from a fallow deer (*Dama dama*) specimen. In line with preliminary studies we exposed cells to 10 μM, 50 μM, 100 μM, 1 mM, and 10 mM ketamine or xylazine. The combination of ketamine-xylazine was dosed at 0.025+0.02 mg/ml, 0.05+0.04 mg/ml, 0.75+0.06 mg/ml, 0.1+0.08 mg/ml, and 0.125+0.1 mg/ml per one well containing 10 000 cells. The quantification of cytotoxicity was based on lactate dehydrogenase activity released from damaged cells.

**RESULTS:** Liver-derived cells show higher sensitivity to the cytotoxic effects of both ketamine and xylazine administered as single drugs when compared with cells cultured from the heart and kidney. The Hellabrunn mixture induced significantly higher cytotoxicity for kidney-derived cells ranging from 16.78% to 35.6%. Single and combined exposures to ketamine and xylazine resulted only in high-dose cytotoxicity in the heart-derived cells.

**CONCLUSIONS:** Our results indicate that immobilization drugs significantly differ in their cytotoxic effects on cells derived from various organs of the fallow deer.

**Abbreviations:**

| | | | |
|---|---|---|---|
| DMEM | - Dulbecco´s Modified Eagle Medium | HSD | - Tukey Honest Significant Difference test |
| FBS | - Fetal bovine serum | LDH | - Lactate dehydrogenase |
| | | PBS | - Phosphate-buffered saline |

## INTRODUCTION

Wildlife immobilization and capture is said to be an art (Fowler 1986). Contrary to that of domestic animals and pets, the outcome depends on many factors and conditions which the veterinarian is poorly able to control. For example, wild animals cannot be restrained prior to immobilization and their body mass can only be estimated to prepare the anaesthetic dose. The wild animal's condition of health is unknown at the moment of capture because there are no clinical examinations, blood chemistry and haematology test results available to select proper drugs (Marco & Lavin 1999). Wildlife immobilizing agents are required to show minimal side effects, to be easy to administer via a small darted volume, and to act fast. The most important quality of drugs used for wildlife anaesthesia, however, is the high therapeutic index or safety ratio (DelGiudice *et al.* 2005). All these properties can be tested on laboratory animals representing *in vivo* experimental models. Problems arise with the extensive testing of the effects of a variety of drugs on a wide range of wildlife animal species. While knowledge about their responses to the anaesthetics are poorly known or even anecdotal, data about the anaesthetic risks associated with wildlife species and certain drugs are important decision-making issues before the immobilization of wildlife animals (Radio & Mundy 2008).

Over the last few decades, the anaesthesia of wildlife species has improved rapidly by using new effective therapeutics. To immobilize wild animals, one has to consider aspects important for the selection of appropriate drugs and their combinations such as the route of administration, concentration and volume of the substance, duration and quality of anaesthesia, potential side effects and availability of antagonists (Machin 2007; Turner *et al.* 2011). Most approved therapeutics do not list wild species on the labelling, meaning that use of these drugs on wildlife is often extra-label and may pose increased anaesthetic risks (AMDUCA 1994). The pharmacodynamics of anaesthetics in wildlife species is poorly understood because of limited dose-response research data (Lees *et al.* 2004).

Some common side effects of anaesthetics include muscle tremors, blood pressure alteration, and respiratory and cardiovascular depression. Adverse reactions resulting in agitation and hallucination may be observed as the action of the medication fades away (Hall *et al.* 2001).

*In vitro* models of wildlife species-derived cell cultures are a cutting-edge tool of biomedical research (He *et al.* 2014) and may also be useful for toxicological screening to facilitate decisions in choosing therapeutics for different animal species.

Various chemical substances based on opioids, cyclohexamine or α2 adrenergic receptor agonists such as ketamine (Hall *et al.* 2001), xylazine (Greene 1988) or a combination of ketamine and xylazine (Galka *et al.* 1999) are currently used to immobilize wild animals. New combinations of drugs like butorphanol-azaperone-medetomidine (Miller *et al.* 2009) or nalbuphine-medetomidine (Wolfe *et al.* 2014) are also being tested.

Ketamine produces a dose-related state of unconsciousness and analgesia referred to as dissociative anaesthesia. Ketamine-induced analgesia could also be related to a functional interaction with opioid receptors (Kohrs & Durieux 1998). Testing on animals confirmed the different effects of ketamine isomers on the central nervous system and a growing body of evidence indicates that ketamine can cause neurotoxicity in a variety of animal models. Recent studies on anaesthetics have shown that clinically relevant doses of ketamine are able to trigger massive and widespread apoptotic neurodegeneration in the immature rat brain (Ikonomidou *et al.* 1999; Scallet *et al.* 2004). Apart from affecting the central nervous system, anaesthetics also have important side effects on the cardiovascular system. It has been reported that the administration of ketamine can increase the heart rate, blood pressure and cardiac output (Chen *et al.* 2005; Reich & Silvay 1989; Traber *et al.* 1971), but these *in vivo* findings may not be consistent with the findings reported in several models *in vitro* (Brown *et al.* 2015; Lewis *et al.* 2001). The consensus therefore is that ketamine stimulates cardiac function indirectly by altering the autonomic nervous system and directly depresses cardiac function (Rodrigues *et al.* 2006; Saha *et al.* 2007; Wolfe *et al.* 2014).

Xylazine could be a confounding factor because it is an α2 adrenergic receptor agonist and this class of drug has demonstrated the ability to down-regulate pro-inflammatory cytokine expression. Stimulation of α2 adrenergic receptors in the heart leads to vasoconstriction which might result in ischemic preconditioning of the tissue. Sloan *et al.* (2011) speculate that higher doses of xylazine may be responsible for cardioprotective effects. More studies are needed to determine if ketamine and xylazine, administered separately, are able to contribute to cardioprotection or whether higher doses of both agents are necessary. The effect of ketamine/xylazine to abrogate volatile anaesthetic cardioprotection had only been demonstrated via *in vitro* cell models (Kawano *et al.* 2005; Zaugg *et al.* 2002) or suggested based on *in vivo* association between the use of these drugs and ischemic preconditioning and reperfusion (Mullenheim *et al.* 2001; Nakae *et al.* 2000).

The present study aimed to examine the adverse cytotoxic effects of the most commonly used anaesthetic agents in fallow deer, an important game ungulate kept on farms, in enclosures as well as in free hunting grounds.

## MATERIALS AND METHODS

### *Cell isolation*

Liver-, heart- and kidney-derived cell cultures for experiments were prepared from a fallow deer (*Dama*

*dama)* male specimen. Tissue samples were collected immediately after killing the farmed animal and loaded in normal saline supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). Tissues were loosened mechanically with blades, minced into small pieces, suspended in Dulbecco´s Modified Eagle Medium (DMEM) (Biosera, Boussens, France) containing 1 mg/ml collagenase (Thermo Fisher Scientific, Waltham, MA, USA) and 1 mg/ml trypsin (Sigma-Aldrich, St. Louis, Missouri, USA), and then incubated at 37 °C on a shaking thermoblock (Biosan, Riga, Latvia) for 45 min. After these procedures, the cells were separated through a 100 µm nylon filter. Dispersed cells were then washed twice in a medium supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, Missouri, USA) to stop enzymatic digestion. Adherent cells were cultivated for 8 days.

*Drugs and other materials*

To study the cytotoxicity of anaesthetic drugs, we used ketamine hydrochloride (Narketan inj a.u.v., 100 mg/ml, Vétoquinol s.r.o., Nymburk, Czech Republic) and xylazine (Xylased 500 mg lyophilized, Bioveta, Ivanovice na Hane, Czech Republic). These substances were either used directly or diluted in a medium for experiments (see below in the Drug treatment description). The Hellabrunn mixture was prepared by dissolving the lyophilized powder of xylazine with 10% injection solution of ketamine, resulting in the ratio of 100 mg of ketamine to 125 mg xylazine per 1 ml. Different exposure concentrations were then prepared from this stock solution using the addition of a medium for experiments (see below in the Drug treatment description).

*Drug treatment*

Cells isolated for the experiments were cultured using DMEM/F12 1:1 medium (Biosera, Boussens, France) (supplemented with 10% FBS, L-glutamine, 15 mol/l Hepes, penicillin 100 IU/ml, and streptomycin 100 µg/ml) in a 96-well plate at 37 °C under a humidified atmosphere of 5% $CO_2$ overnight. Optimum cell concentrations for the assay were determined in a preliminary experiment at 10 000 cells per well. Prior to the addition of the experimental solutions, cells were washed with Phosphate-buffered saline (PBS) buffer and nonadherent cells were removed. Ketamine solutions (10 µM, 50 µM, 100 µM, 1 mM and 10 mM concentrations), xylazine solutions (10 µM, 50 µM, 100 µM, 1 mM and 10 mM concentrations) and ketamine-xylazine solutions, i.e. the Hellabrunn mixture (dosed at 0.025+0.02 mg/ml, 0.05+0.04 mg/ml, 0.75+0.06 mg/ml, 0.1+0.08 mg/ml and 0.125+0.1 mg/ml) were prepared immediately prior to the experiment. Cells were incubated under exposure of tested substances in the dark at 37 °C for 2 h. The medium for the experiments was DMEM with L-glutamine, without pyruvate (Biosera, Boussens, France) supplemented with 1% FBS.

*Cytotoxicity evaluation*

The colorimetric assay was based on measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells by Cytotoxicity Detection Kit (Roche, Basel, Switzerland). Cytotoxicity was estimated according to the manufacturer's recommendation and recorded using an ELISA reader ELx808 (BioTek, VT, USA).

To compare the differences in cytotoxicity, Statistica for Windows® 10 (StatSoft, Inc., Tulsa, OK, USA) was employed for procedures of the one-way analysis of variance (ANOVA) and post-hoc analysis of means by the Tukey Honest Significant Difference (HSD) test. The levels of significance used were either $p<0.05$ or $p<0.01$.

## RESULTS

The comparative effects of the drugs and responses of specific organ-derived cells from the fallow deer are shown in Figures 1 to 3 and 4 to 6, respectively. While the exposure of kidney- and heart-derived cells

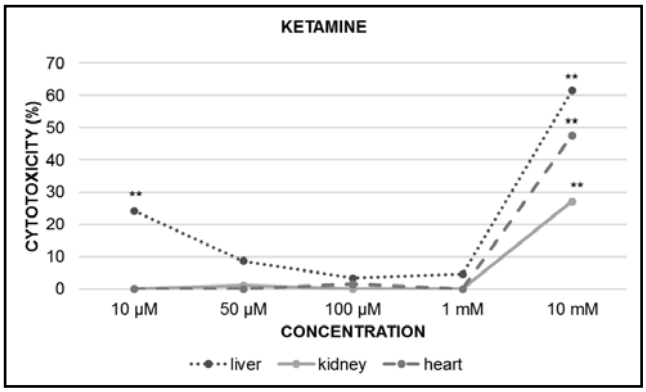


**Fig. 1.** Cytotoxicity of ketamine for liver-, kidney-, and heart-derived cells from fallow deer. Cells were treated with a range of ketamine concentrations from 10 µM to 10 mM, ** = $p<0.01$ when compared against control group (data not shown).

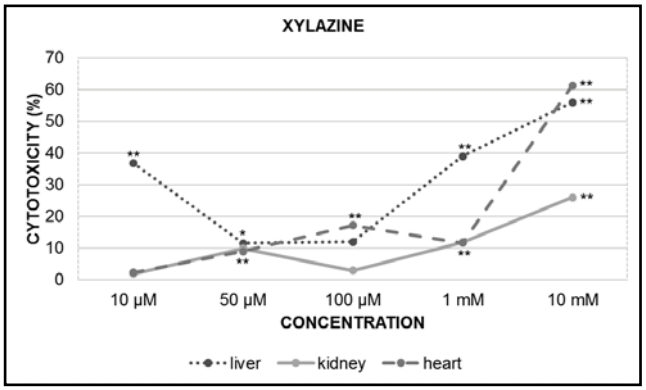


**Fig. 2.** Cytotoxicity of xylazine for liver-, kidney-, and heart-derived cells from fallow deer. Cells were treated with a range of ketamine concentrations from 10 µM to 10 mM, * = $p<0.05$, ** = $p<0.01$ when compared against control group (data not shown).

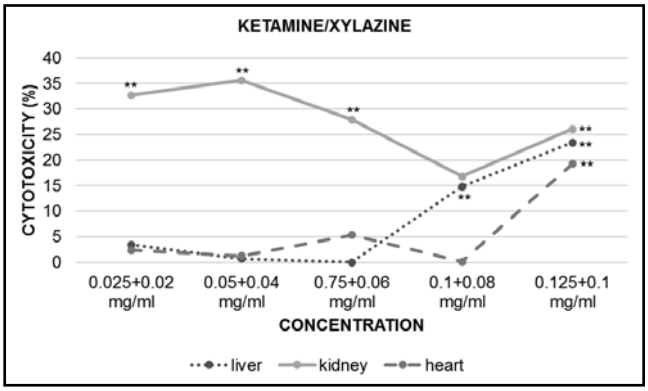


**Fig. 3.** Cytotoxicity of Hellabrunn mixture (100 mg of ketamine plus 125 mg xylazine per 1 ml of DMEM medium) for liver-, kidney-, and heart-derived cells from fallow deer. Cells were treated with an increasing range of fixed ketamine-to-xylazine-ratio concentrations, ** = $p<0.01$ when compared against control group (data not shown).

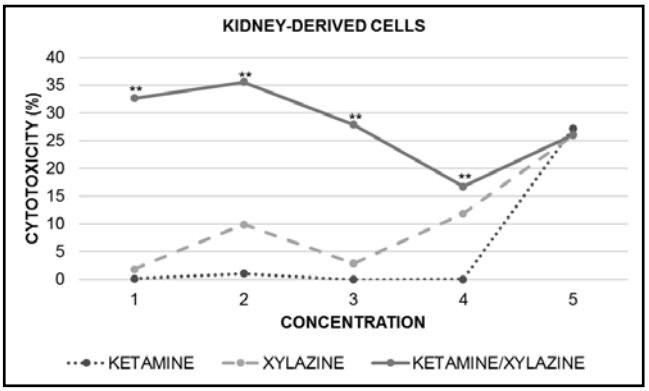


**Fig. 5.** Cytotoxicity of ketamine, xylazine and Hellabrunn mixture for fallow deer kidney-derived cells treated with a range of concentrations of these anaesthetic agents from 10 µM to 10 mM. Effects of anaesthetic agents were compared within each concentration group. Significance (**= $p<0.01$) documents difference between agents showing the highest and lowest cytotoxicity. Groups are designated 1, 2, 3, 4, 5 meaning 10 µM or 0.025+0.02 mg/ml, 50 µM or 0.05+0.04 mg/ml, 100 µM or 0.75+0.06 mg/ml, 1 mM or 0.1+0.08 mg/ml, 10 mM or 0.125+0.1 mg/ml for ketamine and xylazine or Hellabrunn mixture, respectively.

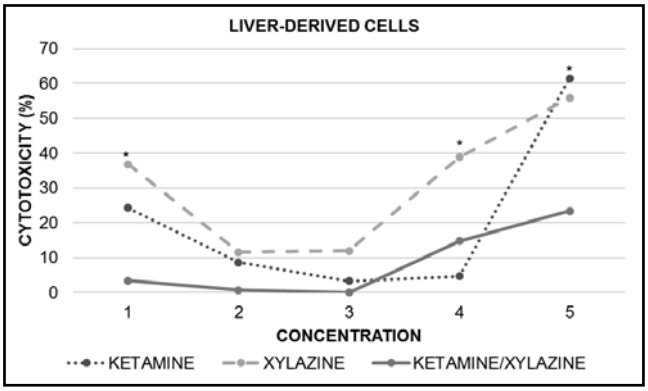


**Fig. 4.** Cytotoxicity of ketamine, xylazine and Hellabrunn mixture for fallow deer liver-derived cells treated with a range of concentrations of these anaesthetic agents from 10 µM to 10 mM. Effects of anaesthetic agents were compared within each concentration group. Significance (*= $p<0.05$) documents difference between agents showing the highest and lowest cytotoxicity. Groups are designated 1, 2, 3, 4, 5 meaning 10 µM or 0.025+0.02 mg/ml, 50 µM or 0.05+0.04 mg/ml, 100 µM or 0.75+0.06 mg/ml, 1 mM or 0.1+0.08 mg/ml, 10 mM or 0.125+0.1 mg/ml for ketamine and xylazine or Hellabrunn mixture, respectively.

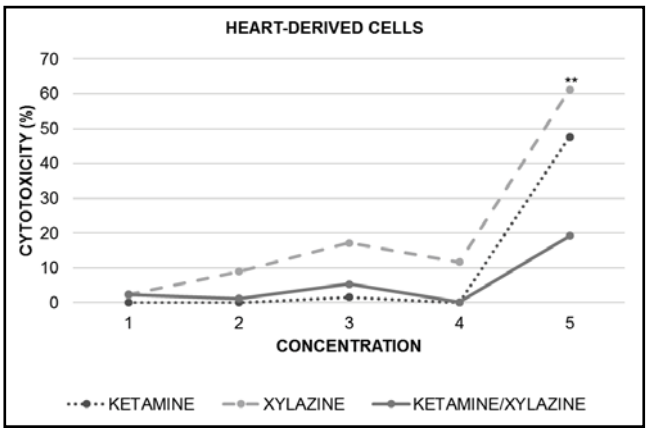


**Fig. 6.** Cytotoxicity of ketamine, xylazine and Hellabrunn mixture for fallow deer heart-derived cells treated with a range of concentrations of these anaesthetic agents from 10 µM to 10 mM. Effects of anaesthetic agents were compared within each concentration group. Significance (**= $p<0.01$) documents difference between agents showing the highest and lowest cytotoxicity. Groups are designated 1, 2, 3, 4, 5 meaning 10 µM or 0.025+0.02 mg/ml, 50 µM or 0.05+0.04 mg/ml, 100 µM or 0.75+0.06 mg/ml, 1 mM or 0.1+0.08 mg/ml, 10 mM or 0.125+0.1 mg/ml for ketamine and xylazine or Hellabrunn mixture, respectively.

to ketamine resulted only in high-dose cytotoxicity, liver-derived cells showed both significant low- and high-dose toxicity when treated with the tested range of ketamine (cf. Figure 1). A similar cytotoxic low- and high-dose-response was observed in liver-derived cells exposed to xylazine (Figure 2). The Hellabrunn mixture of ketamine and xylazine showed significantly higher cytotoxicity for kidney-derived cells ranging from 16.78% to 35.6% across the whole range of treatments when compared with cells from the heart and liver (Figures 3 and 5). Liver-derived cells are more sensitive to the cytotoxic effects of both ketamine and xylazine administered as single drugs when compared with cells cultured from the heart and kidney (Figures 1, 2 and 4). Single and combined exposures to ketamine and xylazine induced only high-dose cytotoxicity in the heart-derived cells (Figure 6).

## DISCUSSION

The present study employed cellular *in vitro* models to examine cytotoxicity of commonly used anaesthetic agents and to extrapolate the risks of immobilization in fallow deer, a wildlife species which is also farmed and kept in enclosures. With respect to the complex-

ity of *in vitro* systems to reproduce tissues and interactions between them in the living animal, the toxicity observed in liver-, kidney- and heart-derived cells can only be used to indicate possible hepatotoxicity, nephrotoxicity and cardiotoxicity, respectively, depending on drug tissue concentrations.

Cell exposure of 2h in the present test corresponded with the standard immobilization duration of game deer species for veterinary procedures. Likewise, cells were treated with the range of ketamine and/or xylazine concentrations from 10 μM to 10 mM based on evidence from *in vivo* animal studies (Bosnjak *et al.* 2012) and data documenting ketamine levels that maintain anaesthesia (McLean *et al.* 1996).

Our results provide data from *in vitro* experiments on cell cultures, which might sway decisions in anaesthetic choice and dosage for fallow deer. First, the Hellabrunn mixture of ketamine and xylazine proved to be cytotoxic to fallow deer kidney cells independent of dosage. Utilization of the Hellabrunn mixture might thus be discouraged in cases indicative of nephropathology. Renal support should also be the mainstay of the treatment of patients under the Hellabrunn mixture anaesthesia. While anaesthetics are commonly combined to enhance some of their qualities and decrease their dosage, it has already been shown that combined exposure to multiple toxins promotes some of their adverse effects and that co-exposures in sub-lethal doses may cause different effects than single substance exposures (Bandouchova *et al.* 2011; Ondracek *et al.* 2012, 2015; Osickova *et al.* 2012, 2014; Pikula *et al.* 2010). Secondly, while an overdose logically proved cytotoxic in our experiment with all three organ-derived models, the significant low-dose toxicity in liver-derived cells treated with ketamine or xylazine stresses the importance of relating dose to body mass also at the other end of the dosage spectrum. The discontinuity between low- and high-dose cytotoxicity of ketamine and xylazine for liver-derived cells suggests that multiple physiological mechanisms respond to the drug exposure. Since our results indicate the critical importance of assessment of the animal's weight prior to its anaesthesia, the rule to double the administered dose in open hunting grounds, when fast action of anaesthetics is required to avoid loss of the animal in the field, seems risky for the animal's health and life. Considering the hepatotoxicity, supportive therapy with hepatoprotective drugs is warranted in deer specimens immobilized with ketamine and xylazine.

One of the most important complications of the capture and handling free-ranging deer is capture myopathy (Paterson 2007). Based on our results of high-dose cytotoxicity observed in the heart-derived cells, it may be hypothesized that these drug-related effects might act as factors contributing to the exertional skeletal and cardiac muscle rhabdomyolysis and exacerbate the problem. On the other hand, higher doses of ketamine and xylazine mixture were shown to decrease the cardiac damage in a guinea pig ischemia-reperfusion model (Sloan *et al.* 2011). Developmental neurotoxicity (Scallet *et al.* 2004, Vutskits *et al.* 2008) of ketamine is another aspect to be considered and avoided when planning the wildlife immobilization of females that might be pregnant. Vutskits *et al.* (2008) suggest the dose-dependent ability of ketamine to induce cell death; even its very low concentrations may interfere with dendritic arbour development and the survival of immature GABAergic neurons.

## CONCLUSION

To conclude, understanding the possible adverse effects associated with the use of the studied anaesthetics in fallow deer are essential to decrease and prevent capture-related complications and mortality.

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