Diclofenac-Induced Cytotoxicity in Cultured Carp Leukocytes

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Summary

Diclofenac is a drug commonly used in human and veterinary medicine for the treatment of diseases associated with inflammation and pain. Medicinal products enter waste and surface waters on an everyday basis and contaminate the aquatic environment. Fish are therefore permanently exposed to these chemicals dissolved in their aquatic environment. To simulate variable environmental conditions, the aim of our study was to examine adverse effects of diclofenac under different temperatures of cell incubation (18, 21, 24, 27 and 30 °C). Cyto-toxic and -static effects of diclofenac in concentrations of 0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml for the carp (*Cyprinus carpio*) cultured leukocytes were quantified using detection of lactate dehydrogenase released from damaged cells. Overall DCF cytotoxicity was relatively low and its impact was pronounced at higher temperature and DCF concentration. Cells growth inhibition is changing more rapidly but it is high mainly at the highest concentration from low temperature. DNA fragmentation was not detected in tested leukocyte cell line. CYP450 increased diclofenac cytotoxicity only at the highest concentration but at incubation temperatures 18 and 27 °C. Leukocyte viability is essential for immune functions and any change can lead to reduction of resistance against pathogens, mainly in cold year seasons, when the immune system is naturally suppressed.

Key words

Fish • Immune system • Non-steroidal anti-inflammatory drug • Toxicity • Temperature variation • Environmental pollution

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Introduction

Human and wildlife health and disease are intricately interlinked (Forget and Lebel 2001, Zinsstag *et al.* 2011). Development and use of efficient drugs improves the quality of human and animal life. On the other hand, industrially produced medicinal products are mostly non-natural, hence, adverse effects from environmental drug residues may be a serious cause for concern *(*Arnold *et al.* 2013, Hruba *et al.* 2019, Mezzelani *et al.* 2016). Pharmaceutica*ls* can influence aquatic organisms as well as contaminate ground and drinking water sources and other parts of ecosystems.

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is amongst the most widely prescribed medicines worldwide and, consequently, one of the most frequently detected compounds in waste and surface waters (McGettigan and Henry 2013, Schwaiger *et al.* 2004). It is used in the treatment of pain, physical disability in rheumatic diseases as well as for its anti-inflammatory and antipyretic effects (Kołodziejska and Kołodziejczyk 2018). In general, diclofenac, as a non-selective cyclooxygenase (COX) inhibitor, acts on both types of COX, namely COX-2 synthesized during inflammatory processes (therapeutic effect) and COX-1 with physiological function in synthesis of prostaglandins, which are important for protection of gastrointestinal epithelial cells (Hawkey 2000, Laine

1996), for blood pressure balance, correct thrombocyte aggregation and some other cardiovascular system characteristics (Al-Saeed 2011, Grosser *et al.* 2006). Therefore, adverse effects are commonly associated with the above-mentioned systems (Al-Saeed 2011, Laine 1996). Importantly, however, detoxification and elimination pathways of xenobiotics including diclofenac are located in the liver and kidney, where diclofenac can be concentrated and/or metabolized by the enzyme complex of cytochrome P450 (CYP450) to form toxic metabolites (Boelsterli 2002, Shen *et al.* 1999, Tang *et al.* 1999). Nephro- (Hickey *et al.* 2001, Murray and Brater, 1993) and hepato-toxicity (Aithal and Day 2007, Helfgott *et al.* 1990) can be observed consequently.

The primary source of drugs and their metabolites in the environment are patients. The active substances and/or their metabolites are excreted in faeces and urine. First, pollutants contaminate wastewater treatment plants where they should ideally be eliminated. However, imperfectly captured micropollutants pose a risk because "treated" wastewater is released into the environment. Second, drugs used to treat animals can directly get into the soil with manure and later they may be flushed with rainwater. Expired drugs entering the cycle in the form of seepage from landfills is another source of pollution (Lonappan *et al.* 2016, Zuccato *et al.* 2000).

The dose, chemical structure and route of administration are important factors influencing the environmental burden of each biologically active substance (Daughton and Ruhoy 2013). Environmental studies revealed concentrations amounting up to a few hundred nanograms of diclofenac per liter of water. In the Czech Republic, the highest observed value of 1080 ng/l was detected in the Elbe river with relatively high flow rate of more than 60 m^3/s. At the same time, significantly higher levels of diclofenac residues can be detected in smaller watercourses with low flow rate, rural localities with intensive agriculture, in rivers below wastewater treatment plants and in the lower parts of streams (Buser *et al.* 1998, Lacina *et al.* 2013, Marsik *et al.* 2017, Tixier *et al.* 2003, Ternes, 1998).

While diclofenac concentrations detected in water usually do not induce acute toxicity with lethal effects (Lonappan *et al.* 2016), it accumulates in fish organs in a concentration-dependent manner (Schwaiger *et al.* 2004). It was found that fish show abnormal behavior, respiratory distress, changes in biochemical and hematological parameters and pathological alterations in the liver, kidney and gills suggestive of inflammatory and necrotic processes associated with exposure to diclofenac (Ajima *et al.* 2015, Hoeger *et al.* 2005, Schwaiger *et al.* 2004, Stepanova *et al.* 2013), resulting in general health impairment (Schwaiger *et al.* 2004).

The majority of ecotoxicology experiments were designed to examine effects of diclofenac at the level of whole fish organisms (Ajima *et al.* 2015, Hoeger *et al.* 2005, Schwaiger *et al.* 2004, Stepanova *et al.* 2013), maintaining a narrow range of aquarium water parameters including the temperature. Immune defences of non-homeothermic vertebrates are temperature-dependent (Heger *et al.* 2020, Morvan *et al.* 1998). While immunotoxicity of diclofenac in fish is still understudied (Schwaiger *et al.* 2004), fish cell cultures provide useful tools for experimental approaches with temperature variation. Here, we performed diclofenac toxicity testing using cultured carp leukocytes and predicted both higher cytotoxicity rates and proliferation inhibition at lower temperatures. We also aimed to obtain data applicable for environmental pollution risk evaluation of diclofenac in the common carp (*Cyprinus carpio*), an important aquaculture species.

Materials and Methods

Cell line and chemicals

We used common carp (*Cyprinus carpio*) leukocytes cell culture number 95070628 (Merck Sigma-Aldrich, Germany) for diclofenac toxicity testing. Following chemicals were used during cultivation and testing: culture medium Dulbecco's Modified Eagle Medium/Ham's F-12 (1:1), assay medium Dulbecco's Modified Eagle Medium (DMEM) w/o sodium pyruvate, bovine calf serum (BCS) 10 %, antibiotics (ATB) (penicillin 100 IU/ml and streptomycin 100 μg/ml, Biosera, Boussens, France), diclofenac sodium salt (Sigma-Aldrich, Germany), 1A2 Cytochrome P450 human expressed in *Pichia pastoris* (CYP450) (Merck, Sigma-Aldrich, Germany), lactate-dehydrogenase (LDH) activity assay kit (Roche Sigma-Aldrich, Germany), dimethyl sulfoxide (DMSO), phosphate saline buffer, and trypsin solution (Sigma-Aldrich, Germany).

Solutions for treatment of cultures

After reduction of light intensity in the laboratory, diclofenac was solubilized in DMSO and diluted with DMEM w/o sodium pyruvate to obtain final testing concentrations of diclofenac. Two experiments

with different concentrations of diclofenac were performed (see Table 1). The tested concentration range started with 1 ng/ml based on the value detected in the Elbe river (Marsik *et al.* 2017). It can also be assumed that diclofenac concentrations in the aquatic environment can be higher than the observed values due to accumulation of residues in the environment (Marsik *et al.* 2017) and in organs and tissues of fish (Schwaiger *et al.* 2004). Moreover, higher tested concentrations were selected in order to induce distinct cytotoxic effects (cell killing versus growth rate inhibition), suggestive of the fact that leukocytes are more resistant to diclofenac than other cell types (Němcová, 2018).

Cytotoxicity assay

The number of injured cells was assessed through measuring intracellular lactate dehydrogenase leakage from damaged cells using LDH activity assay kit according to the manufacturer's instructions. Absorbance of product of reaction was measured at 490 nm using ELISA reader (Elx808, BioTek, USA). For calculation of cytotoxic and inhibition effects we used modified protocol according to Smith *et al.* (2011).

Detection of DNA fragmentation

After the cultured cell pellet was obtained by centrifugation (1000 rpm for 6 min) of the cell suspension (the medium was removed and cells were detached from the cultivation surface using trypsin solution). DNA for detection of DNA fragmentation was isolated using NucleoSpin® Tissue (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Isolated DNA was then analyzed by electrophoresis in 2.5 % agarose gel (100 V for 90 min, 50 V for 50 min, 35 V for 50 min) after staining with DNA Stain G (SERVA, Heidelberg, Germany) and visualisation on a UV transilluminator (Ultra Lum Inc., Claremont, Canada).

Experiment 1: Influence of diclofenac sodium salt on carp leukocytes

Cells in 96-well plates (10 000 cells/well for 12 h, 8 000 cells/well for 24 and 48 h) were pre-incubated (Benchmark, myTemp™ Mini CO_2, USA) overnight in medium DMEM-F12 supplemented with 10 % BCS and 1 % ATB under humidified atmosphere of 5 % CO_2 at 27 °C. Then the medium was replaced with diclofenac-supplemented or control solutions (see Table 1). The treated cells were incubated (ESCO, CellCulture CO_2, USA) in the dark under humidified atmosphere of 5 % CO_2 at five different temperatures of 18, 21, 24, 27 and 30 °C. To determine cytotoxicity, we detected lactate dehydrogenase released from damaged cells after 12, 24 and 48 h of exposure.

Table 1. Experimental design information

	Experiment 1									
DCF concentration	control with cells (0 µg/ml)	control blank (0 µg/ml)	test blank (100 µg/ml)	0.001 µg/ml	0.01 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	
	*	*	*	c1	c2	c3	c4	c5	c6	
DMSO concentration	not exceeding 0.5 %									

	Experiment 2		
	DCF concentration		
		DCF 0 µg/ml	**DCF 100 µg/ml**
CYP concentration	*CYP450 0 µg/ml*	control with cells	*
	CYP450 0.5 µg/ml	*	*
	CYP450 5 µg/ml	*	*
DMSO concentration	not exceeding 0.5 %		

DCF = diclofenac; DMSO = dimethyl sulfoxide; cytochrome = CYP

Experiment 2: Influence of diclofenac metabolism in carp leukocytes on cytotoxicity

Cells in plates (8 000 cells/well in 96-wells plate for LDH assay or 50 000 cells/well in 6-wells plate for DNA isolation) were pre-incubated overnight in DMEM-F12 supplemented with 10 % BCS and 1 % ATB under humidified atmosphere of 5 % CO_2 at 27 °C. The medium was then replaced with test solutions (Table 1). The treated cells were incubated in the dark under humidified atmosphere of 5 % CO_2 at different temperatures of 18 °C (sub-optimal cultivation temperature, immune response initiated *in vivo*), 27 °C (optimal temperature for cultivation recommended for carp cell cultures by the supplier (Merck Sigma-Aldrich, Germany) and researchers (Chen *et al.* 2009, Liu *et al.* 2015) and 30 °C (temperature over the optimal cultivation temperature range). Cytotoxicity was then measured in 96-wells plates using detection of lactate dehydrogenase released from damaged cells after 24 exposure. The 6-wells plates were used for DNA isolation to detect DNA fragmentation after 48 h of exposure.

Data analysis

Two parameters were calculated separately for any experimental well, i.e. percent killing (cytotoxicity) and percent inhibition according to Smith *et al.* (2011). Normal distribution of variables in each dataset was tested using the Shapiro-Wilk test. All results in Experiment 1 were obtained from five independent measurements but only two to four measurements were obtained during Experiment 2. The influence of categorical predictors (duration of cultivation, temperature of cultivation and diclofenac concentration) and their interactions to both dependent variables was calculated using Multi-Factorial ANOVA. Predictive desirability model was calculated according to Derringer and Suich (1980) with least square fitting. The relationship between cytotoxicity and inhibition values was analyzed by Pearson correlation coefficient. The difference between the CYP450 groups (Experiment 2) was calculated using one-way ANOVA followed by the Scheffe's post hoc test as there was no significant variance in homogeneity. P < 0.05 was considered a significant difference. All analyses were performed in Statistica for Windows®13.2 (StatSoft, Inc., Tulsa, OK, USA).

Results

Experiment 1

The longest incubation period (48 h) was excluded from the analysis because cytotoxicity results were extremely influenced by the increasing inhibition of cell growth (Fig. 1). This negative correlation was significant at all diclofenac concentrations except for 0.1 µg/ml (p = 0.069) (Table 2) diclofenac cytotoxicity was generally low and does not occur within short periods of cell incubation and exposure (12 h). It was increased at temperatures of 24 °C and higher after 24 h of incubation reaching the highest average value of 21 % (diclofenac concentration 100 µg/ml at cultivation temperature 27 °C and 24 h) (Fig. 2). Factorial ANOVA confirmed the influence of all analyzed factors (time, temperature, concentration) but the impact of concentration was not dependent on the other two factors (Table 3). On the contrary, cellular growth inhibition was also significantly influenced by all parameters under study and the whole model explained more than 80 % of variability. Inhibition increased in response to increasing diclofenac concentrations right from the lowest incubation temperature (18 °C) after the shortest incubation time (12 h) (Fig. 3). The different response of both dependent variables was confirmed also by predictive desirability models (Fig. 4).
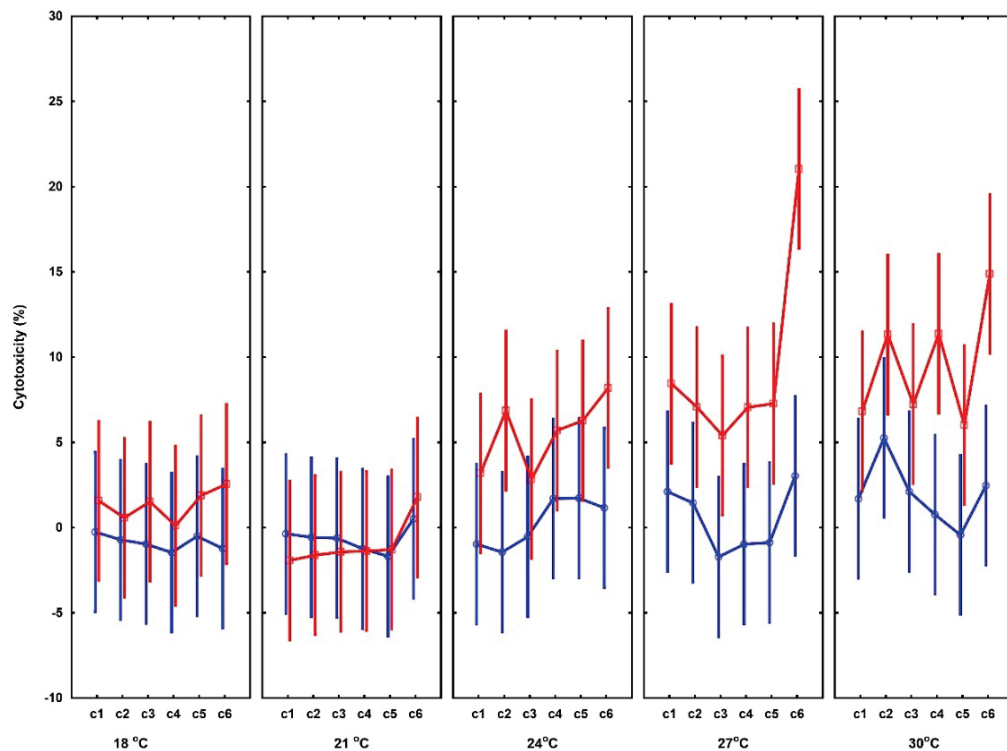
Experiment 2

One-way ANOVA confirmed impact of CYP450 on carp leucocytes at the highest incubation temperature (30 °C) (Table 4). Cytotoxicity of both CYP450 concentrations (0.5 µg/ml and 5 µg/ml) significantly differed from the control group (post-hoc Scheffe's test).

Table 2. Pearson correlation two diclofenac-induced effects (cytotoxicity versus inhibition of growth) in carp (*Cyprinus carpio*) leukocyte cultivated *in vitro* in Experiment 1, after 48 incubation hours.

Diclofenac concentration	r	p
0.001 µg/ml	-0.563	0.003
0.01 µg/ml	-0.700	< 0.001
0.1 µg/ml	-0.370	0.069
1 µg/ml	-0.585	0.002
10 µg/ml	-0.548	0.005
100 µg/ml	-0.599	0.002
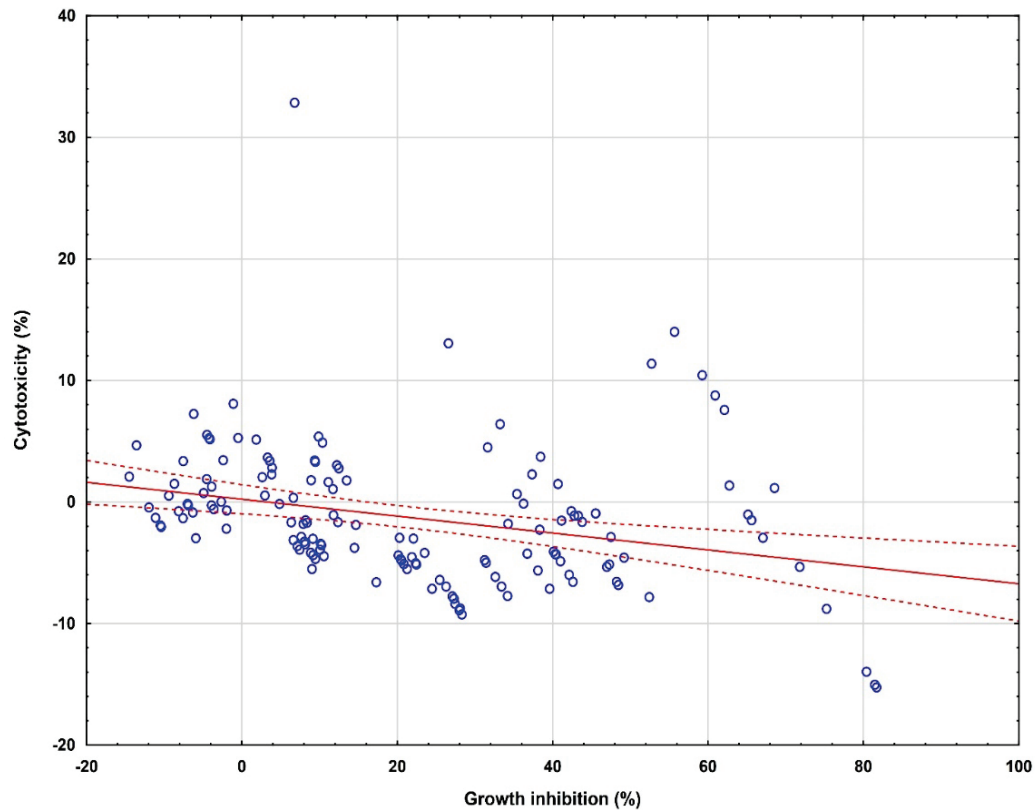
Fig. 1. The dependence of calculated percentage of cell death on growth inhibition level. The correlation between two observed cytotoxic effects (cell death versus inhibition of growth) caused by diclofenac in carp (*Cyprinus carpio*) leukocyte cultivated *in vitro* in Experiment 1, after 48 incubation hours at different temperatures (18, 21, 24, 27 and 30 °C). Cells were treated with various diclofenac concentrations (0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml).
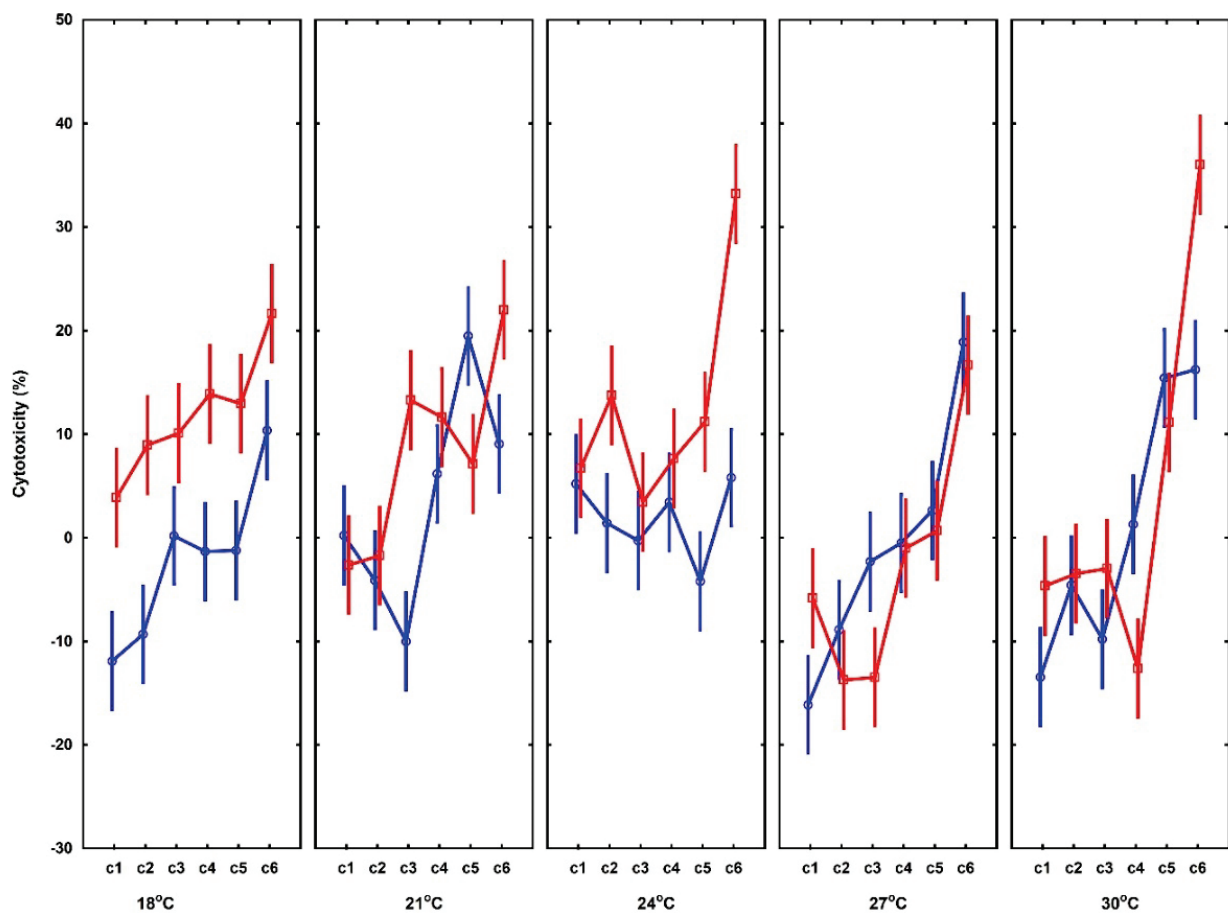

Fig. 2. Diclofenac-induced cytotoxicity in cultured carp (*Cyprinus carpio*) leuko-cytes. The percentage of killed carp leukocytes in Experiment 1, determined after 12 (blue line) and 24 (red line) hours of incubation at different temperatures (18, 21, 24, 27 and 30 °C). Cells were treated with various concentrations diclofenac (c1: 0.001 μg/ml, c2: 0.01 μg/ml, c3: 0.1 μg/ml, c4: 1 μg/ml, c5: 10 μg/ml and c6: 100 μg/ml).

Table 3. Results of factorial analysis of variance (ANOVA) of diclofenac-induced cytotoxic and cytostatic effects.

Effect	DF	Cytotoxicity				Growth inhibiton			
		SS	MS	F	p	SS	MS	F	p
Intercept	1	2040.08	2040.08	72.59	< 0.001	4077.66	4077.66	145.10	< 0.001
Time	1	1679.22	1679.22	59.75	< 0.001	2886.32	2886.32	102.70	< 0.001
Temp	4	1977.17	494.29	17.59	< 0.001	3127.83	781.96	27.83	< 0.001
DCF conc	5	533.21	106.64	3.79	0.003	18221.37	3644.27	129.67	< 0.001
*Time * Temp*	4	864.10	216.03	7.69	< 0.001	2373.84	593.46	21.12	< 0.001
*Time * DCF conc*	5	235.16	47.03	1.67	0.142	1148.07	229.61	8.17	< 0.001
*Temp * DCF conc*	20	585.31	29.27	1.04	0.414	5172.37	258.62	9.20	< 0.001
*Time * Temp * DCF conc*	20	218.12	10.91	0.39	0.992	4387.2	219.36	7.81	< 0.001

Time = duration of cultivation; Temp = temperature of cultivation; DCF conc = diclofenac concentration.

Fig. 3. Diclofenac-induced cytostatic effect in cultured carp (*Cyprinus carpio*) leukocytes. The percentage provides information about growth inhibition level in carp leukocyte cell culture in Experiment 1, determined after 12 (blue line) and 24 (red line) hours of incubation at different temperatures (18, 21, 24, 27 and 30 °C). Cells were treated with various concentrations diclofenac (c1: 0.001 µg/ml, c2: 0.01 µg/ml, c3: 0.1 µg/ml, c4: 1 µg/ml, c5: 10 µg/ml and c6: 100 µg/ml).
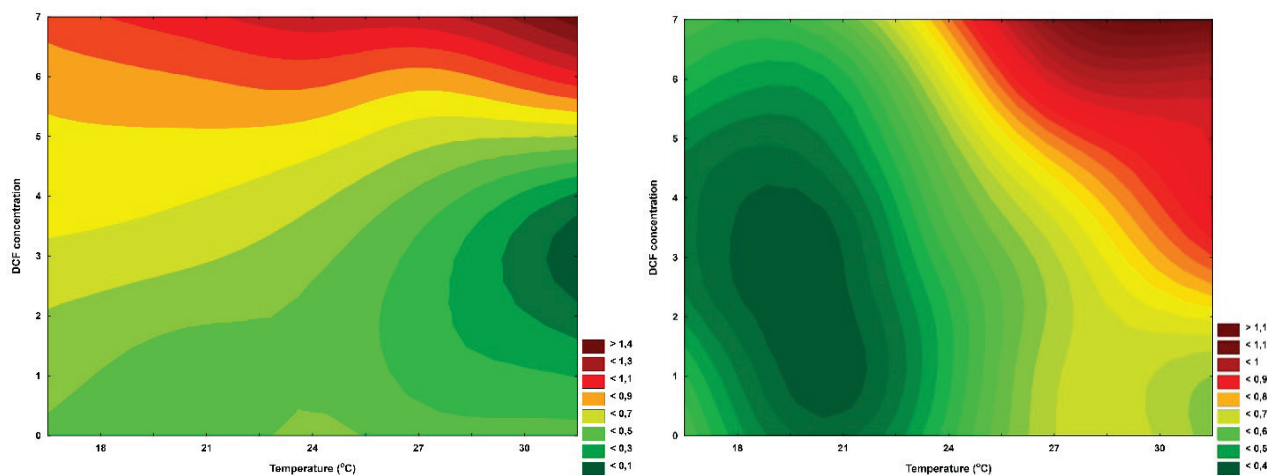
Fig. 4. Contour plots of the levels of overall response desirability for (**a**) diclofenac-induced cytotoxicity and (**b**) diclofenac-induced growth inhibition based on desirability profiling with the least squares fit. There is obvious difference in the response of analyzed parameters to the highest diclofenac concentration i.e. cytotoxic effect only at the highest temperatures but cytostatic effect at all temperatures.

Table 4. Results of one-way analysis of variance (ANOVA) of cytochrome P450 cytotoxic effect.

| | | | Scheffe's post-hoc test | | |
Temperature	F	p	control vs low CYP	control vs high CYP	low CYP vs high CYP
18 °C	3.614	0.084	0.967	0.138	0.122
27 °C	13.539	0.003	0.073	0.176	0.003
30 °C	22.125	< 0.001	0.041	< 0.001	0.018

Control = cells treated only with 100 µg/ml of diclofenac; low CYP = cells treated with 100 µg/ml of diclofenac and 0.5 µg/ml cytochrome P450; high CYP = cells treated with 100 µg/ml of diclofenac and 5.0 µg/ml cytochrome P450.
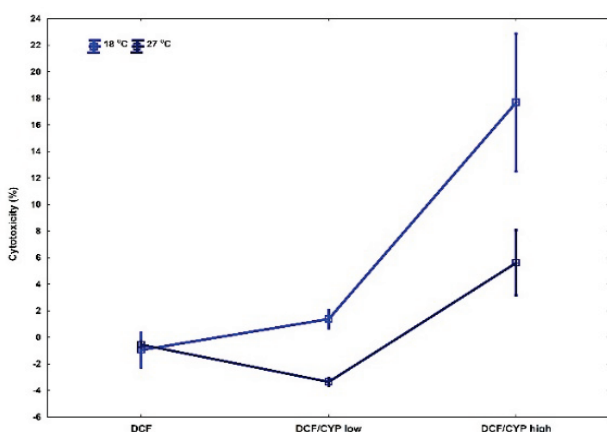
Fig. 5. Cytotoxic effect caused by combination of diclofenac and cytochrome P450 in carp (*Cyprinus carpio*) leukocyte cultivated *in vitro*. The percentage of killed carp leukocytes in Experiment 2, determined after 24 hours of incubation at different temperatures (18 and 27 °C). Cells were treated with 100 µg/ml of diclofenac (DCF) in combination with different cytochrome P450 concentrations (CYP low: 0.5 µg/ml or CYP high: 5 µg/ml).

Therefore, we excluded the experimental group incubated at 30 °C from the subsequent analysis. Combined impact of diclofenac and CYP450 significantly differed at the higher concentration of CYP450. The average cytotoxicity reached the highest values for both incubation temperatures, 17.7 % and 5.6 %, respectively (Fig. 5). DNA fragmentation was not detected in the tested leukocyte cell line following exposure to diclofenac.

Discussion

Leukocytes are an essential part of the immune system playing an important role in defence against pathogenic agents. The number of viable leukocytes is one of key factors for maintaining individual's health. Even a low decrease in viability of these cells can weaken the organism and intensify its susceptibility to disease (Beutler 2004, Desai *et al.* 2010, Neumann *et al.* 2001,

Turvey and Broide 2010). Therefore, our study aimed to investigate the response of cultured carp leukocytes (percentage of viable versus dead cells and growth inhibition) following exposure to diclofenac, a frequent aquatic environment contaminant around the world (Buser *et al.* 1998, Lacina *et al.* 2013, Marsik *et al.* 2017, Tixier *et al.* 2003, Ternes 1998). Moreover, toxicity of diclofenac and any other environmental pollutant may be enhanced by interaction with other anthropogenic xenobiotics or naturally-occurring toxins (Cleuvers 2004, Osickova *et at.* 2014, Paskova *et al.* 2011), posing a threat for wildlife species and whole ecosystems (Owa 2013).

While in fish exposed to diclofenac, the number of white blood cells at organismal level slightly increases (Ajima *et al.* 2015), similar to homeotherms (Lim *et al.* 2006, Martinez *et al.* 1999), little is known about diclofenac effects at the cellular level, despite existence of standardized ethically acceptable *in vitro* methods and fish-derived cell and tissue cultures which may provide scientifically correct targeted results and help understand toxicological processes *in vivo* (May *et al.* 2009, Orbach *et al.* 2017, Wilhelm *et al.* 2011). Our *in vitro* results show that cultured carp leukocytes after short-term (12 h) exposure to diclofenac proliferate slightly (Fig. 2), suggesting an initial response to the drug (Ajima *et al.* 2015), and the percentage of damaged cells during the first approximately 8 h (i.e. necrotic cell death) is low (Niles *et al.* 2009). In general, however, exposure to NSAIDs leads more to apoptosis than necrosis (Ajima *et al.* 2015, Chang *et al.* 2005, Nicotera *et al.* 1999, Niles *et al.* 2009). Therefore, cytotoxicity increases after medium-term (24 h) and longer exposure to diclofenac.

The original LDH-based cytotoxicity protocol grossly underestimates the proportion of dead cells under conditions with cell growth inhibition (Smith *et al.* 2011). We used, therefore, the modified protocol recommended by the above authors (Smith *et al.* 2011), differentiating two cytotoxic effects, i.e. cell death versus growth inhibition. According to this protocol, the calculated percentage of killed cells is still influenced by growth inhibition (Fig. 1). The equation used in the protocol is calculated with "low control values", while non-exposed control cells natural mortality is high, because proliferation of non-exposed cells is significantly higher compared with inhibited test cells as having more cells means more natural deaths (as shown in experiments of longer-term duration 48 h, Fig. 1). In line with this finding, the LDH test as such is not quite relevant for experiments with exposures lasting 48 h and more.

The highest percentage of damaged cells was 21 %, yet, the number of leukocytes in the culture continuously decreased over time (Fig. 3 and 4). These results suggest that diclofenac inhibits cell proliferation (Chang *et al.* 2005, 2006) rather than induces cell destruction by necrosis or apoptosis. Importantly, at temperatures lower than the optimal cultivation temperature range of 24 - 28 °C recommended for carp cell cultures (Chen *et al.* 2009, Liu *et al.* 2015), proliferation of both exposed and non-exposed control cells is reduced by the low temperature (Carey *et al.* 2003, Stephenson, 1966). Likewise, many physiological processes are influenced by the body temperature corresponding with the ambient temperature in poikilotherm fish. Low temperatures suppress immune responses (Bly and Clem, 1991), cell proliferation, differentiation, tissue remodelling and generally growth rate (Harding *et al.* 2016, Neuheimer *et al.* 2011). Thus, renewal of leukocytes is generally slow at low temperatures.

Cytotoxic (Bort *et al.* 1999, Ponsoda *et al.* 1995, Syed *et al.* 2016) and cytostatic (van Leeuwen *et al.* 2011) effects were reported to be influenced by diclofenac metabolisation mediated by CYP P450. Cytochrome P450 enzyme complex addition combined with exposure to diclofenac, however, had no significant influence on the performance of cultured carp leukocytes in the present study, probably due to the fact that blood leukocytes are not primarily active in detoxication of xenobiotics (Filkins 1971) and may not be able effectively utilize CYP P450 to produce cytotoxic metabolites. On the other hand, the combination of high concentration of CYP P450 and diclofenac had a significantly higher cytotoxic effect than other conditions tested in our experiment 2 (Fig. 5). At the surfeit of CYP P450, leukocytes start work with CYP P450 either as a catalyst of detoxifying reactions or the high level of CYP P450 acts as a xenobiotic (cf. temperature 30 °C, Table 4). Understandably, resistance/ susceptibility of leukocytes and other organs/tissues- derived cells to adverse effects of xenobiotics, including diclofenac, will be variable under *in vitro* conditions (Němcová 2018). Likewise, *in vivo* studies show that cell damage by diclofenac-induced oxidative stress is significantly lower in blood compared with liver and/or gill cells (Islas-Flores *et al.* 2013). The oxidative stress is known to affect one of the pathways of leukocyte apoptosis. A possible pathological mechanism is associated with reactive oxygen species-induced DNA damage (Krammer *et al.* 2007, Krüger and Mooren

2014). While DNA fragmentation due to diclofenac-induced oxidative stress was not detected in our experiment with cultured carp leukocytes, oxidative stress induced by diclofenac coupled with massive DNA fragmentation as early as 24 h after exposure to diclofenac in other cell types (Hickey *et al.* 2001). Activated leukocytes develop a certain degree of apoptosis resistance to decrease their cell population damage (Krammer *et al.* 2007, Krüger and Mooren 2014).

To conclude, exposure to diclofenac resulted in reduction of leukocyte numbers through inhibition of cell proliferation and decreasing their viability *in vitro*. Environmental contamination by diclofenac represents, therefore, a burden for the immune system of fish. While cell culture experimental models are useful for identifying and understanding environmental pollution problems in line with the 'Three Rs' concept of animal welfare and protection against cruelty, extrapolation of cellular effects for the whole organism *in vivo* is still a challenge (Abdelsalam *et al.* 2020, Kovacova *et al.* 2016).

Conflict of Interest

There is no conflict of interest.

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