

Ketoconazole increases the endocrine disrupting potential of ibuprofen exposure in the H295R cells and Japanese medaka



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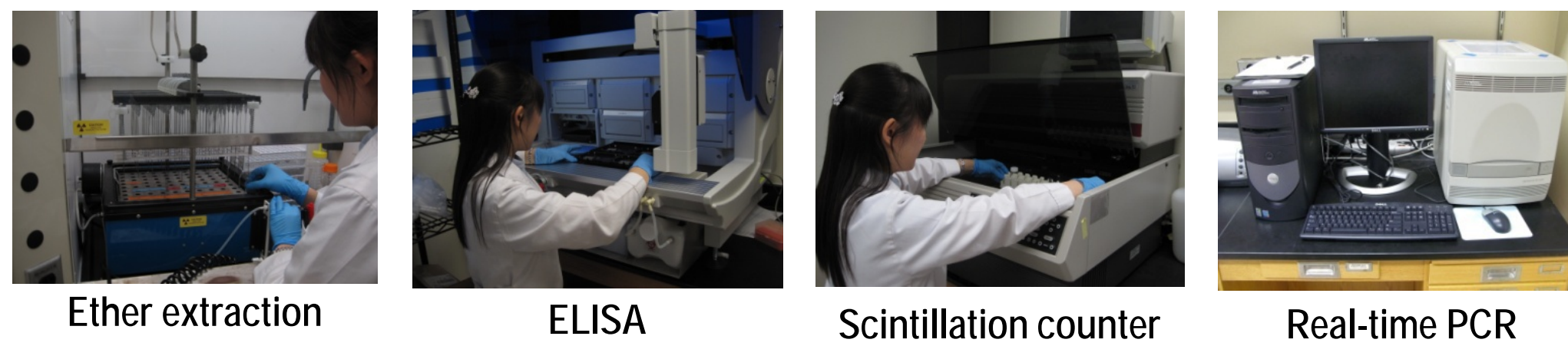
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Introduction

- Pharmaceutical residues found in the aquatic environment occur as mixtures, while most of toxicity tests were made on individual chemicals
- In the present study, we investigated whether exposure to low level ketoconazole would increase the estrogenicity of ibuprofen exposure

Materials & Methods

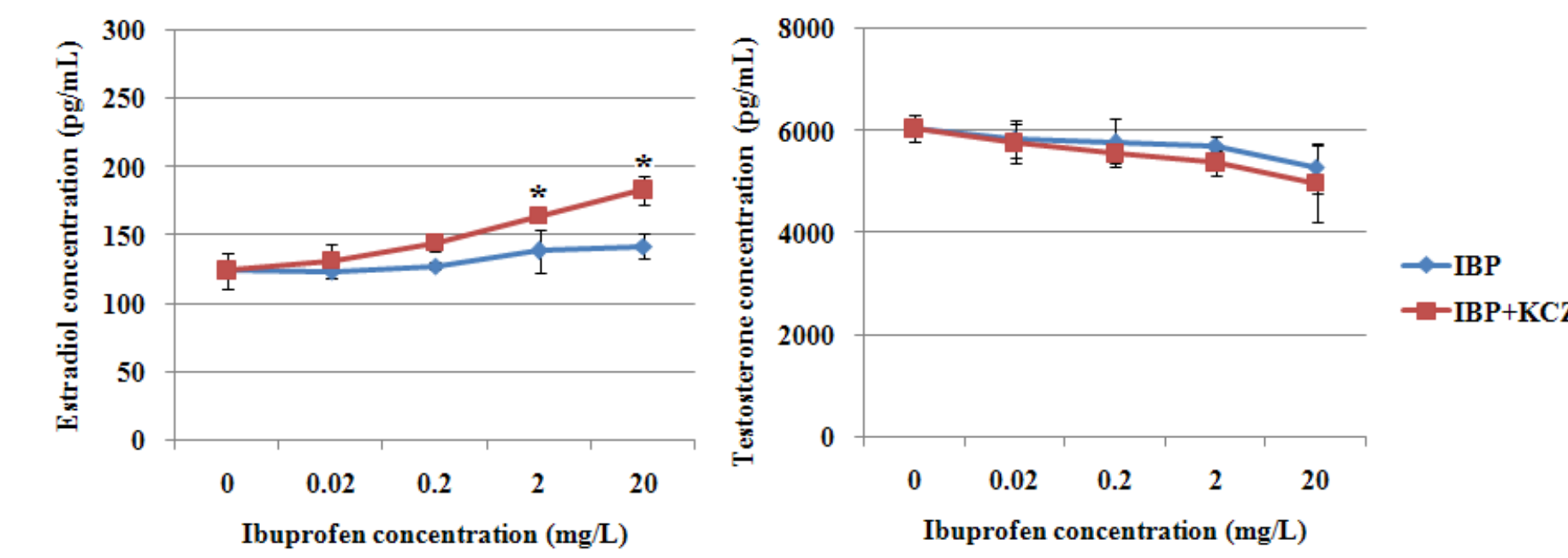
- In vitro H295R cell bioassay**
 - H295R cells were exposed to ibuprofen alone at concentrations ranging from 0.02 to 20 mg/L, or in combination with ketoconazole (5 ng/L) for 48 hr.
 - Hormone measurements
 - Culture medium was extracted twice with 2.5 ml diethyl ether, and hormones were measured by enzyme-linked immunosorbent assay.
 - Aromatase activity assay
 - Direct and indirect effects on aromatase activity were measured by the rate of conversion of 1 β -³[H]-androstenedione to estrone using liquid scintillation counter.
 - Quantitative PCR assay
 - Transcription of five steroidogenic genes (3 β HSD2, CYP11 β 2, CYP17, CYP19, and 17 β HSD) plus one housekeeping gene (β -actin) were measured using real-time PCR.



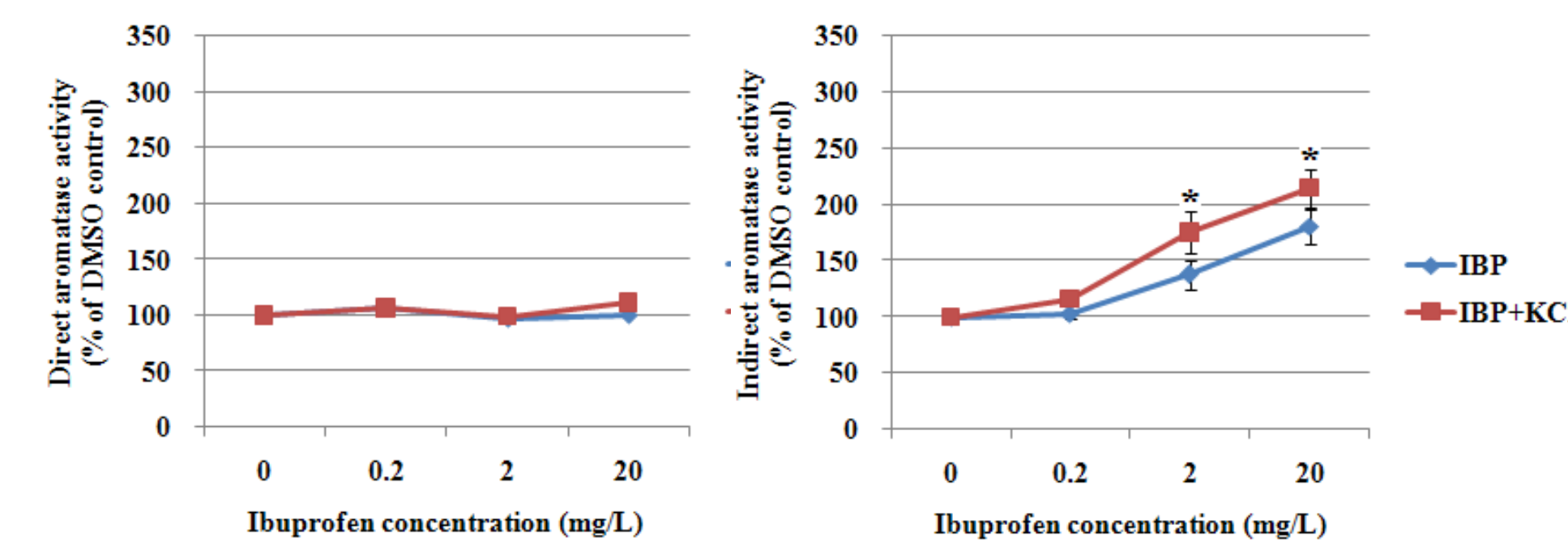
- In vivo Japanese medaka exposure**
 - Male adult medaka (5 fishes/group) were exposed to ibuprofen alone at concentrations ranging from 0.02 to 0.2 mg/L or with ketoconazole (10 μ g/L) for 14 d.
 - On day 14, all surviving fish were euthanized for measurement of sex hormones and related mRNA expressions.

Results & Discussion

- In vitro H295R cell bioassay**
 - 17 β -estradiol (E2) and testosterone (T) measurements
 - Exposure to ibuprofen resulted in significant increase of E2.
 - In combination with ketoconazole, E2 production by ibuprofen exposure was more elevated.



- Aromatase activity assay**
 - Exposure to ibuprofen resulted in significant increase of indirect aromatase activity.
 - When 5 ng/L of ketoconazole was added, the extent of increase in aromatase activity became greater, i.e., up to 1.5-fold compared to ibuprofen only exposure.
 - However, direct aromatase activity did not change.

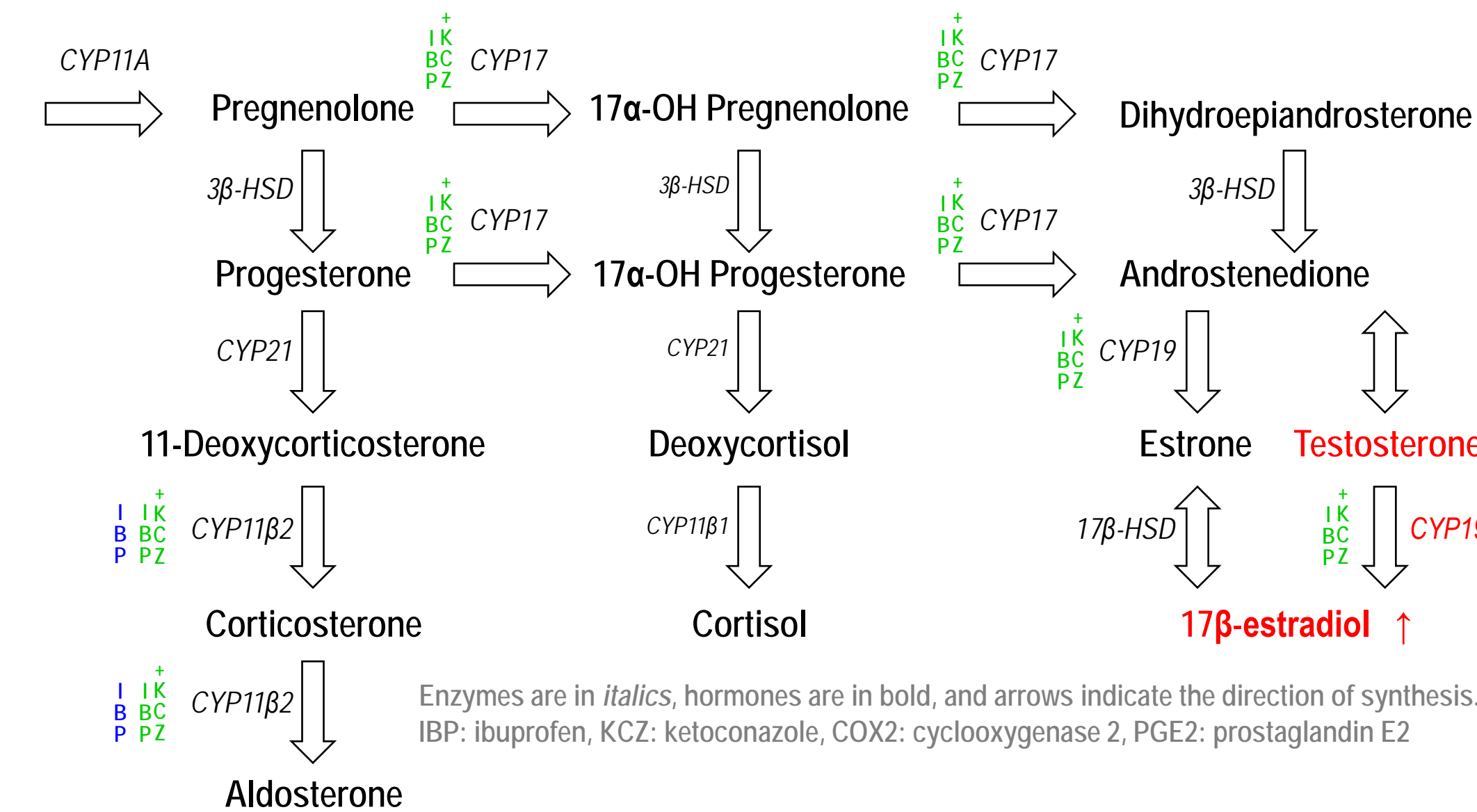


- Quantitative PCR assay**
 - In cells exposed to ibuprofen, the significant differences in CYP11B2 mRNA expression were observed.
 - However, with a combined exposure to ibuprofen and ketoconazole resulted in an elevated expression of CYP17, CYP19, and CYP11B2 mRNAs compared to ibuprofen exposure alone.

Gene	DMSO	IBP		KCZ		IBP+KCZ (5 ng/L)	
	0.1%	0.2 mg/L	2 mg/L	5 ng/L	0.2 mg/L	2 mg/L	2 mg/L
CYP17	1.00 \pm 0.28	1.27 \pm 0.43	1.93 \pm 1.16	0.78 \pm 0.11	2.36 \pm 1.15	3.60 \pm 2.88 *	
CYP19	1.00 \pm 0.30	1.39 \pm 0.25	2.07 \pm 1.02	0.99 \pm 0.18	2.08 \pm 0.96 *	2.98 \pm 0.88 *	
3 β HSD2	1.00 \pm 0.18	0.76 \pm 0.20	0.60 \pm 0.13	0.83 \pm 0.13	0.76 \pm 0.41	0.70 \pm 0.30	
CYP11B2	1.00 \pm 0.46	1.67 \pm 0.94	2.85 \pm 1.16 *	1.33 \pm 0.24	2.49 \pm 1.18	4.38 \pm 1.14 *	
17 β HSD	1.00 \pm 0.70	0.95 \pm 0.12	0.81 \pm 0.60	1.26 \pm 0.25	0.84 \pm 0.23	0.73 \pm 0.45	

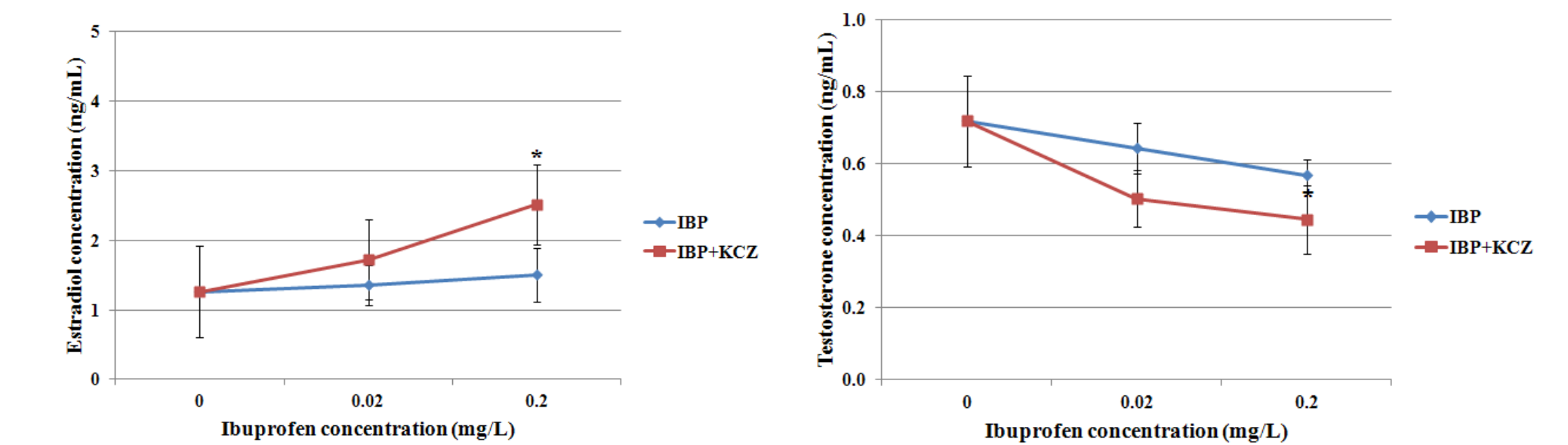
One-way analysis of variance (ANOVA) with Dunnett's test was performed using SPSS 15.0 for Windows[®]. Differences with $p < 0.05$ were considered significant.

- Mechanisms of ibuprofen and ketoconazole exposure**



- In vivo Japanese medaka exposure**

- E2 and T measurements
 - Ibuprofen only did not cause any significant effects on E2 concentration compared to that of control.
 - In combination with ketoconazole, however, E2 production by ibuprofen exposure was more elevated.
 - Significantly lower concentration of T was observed in combination with ketoconazole.



- Quantitative PCR assay**
 - In fish exposed to ibuprofen, no significant differences in mRNA expression were observed.
 - However, with a combined exposure to ibuprofen and ketoconazole resulted in an elevated expression of CYP17 and CYP19 mRNAs compared to ibuprofen exposure alone.
 - Expression of COX2 mRNA was down-regulated in both groups.

Gene	DMSO	IBP		KCZ	IBP+KCZ (10 μ g/L)	
	0.1% (n=6)	0.02 mg/L (n=6)	0.2 mg/L (n=5)	10 μ g/L (n=4)	0.02 mg/L (n=4)	0.2 mg/L (n=6)
CYP17	1.00 \pm 0.26	1.23 \pm 0.25	1.29 \pm 0.20	0.88 \pm 0.24	1.27 \pm 0.31	1.48 \pm 0.22 *
CYP19	1.00 \pm 0.31	1.19 \pm 0.28	1.24 \pm 0.17	0.89 \pm 0.11	1.20 \pm 0.27	1.60 \pm 0.25 *
COX2	1.00 \pm 0.29	0.59 \pm 0.10 *	0.52 \pm 0.12 *	0.93 \pm 0.22	0.63 \pm 0.11 *	0.49 \pm 0.12 *

One-way analysis of variance (ANOVA) with Dunnett's test was performed using SPSS 15.0 for Windows[®]. Differences with $p < 0.05$ were considered significant.

Conclusion

- We have shown that non-effective concentrations of ketoconazole can increase the potential for endocrine disrupting effects of ibuprofen in both human adrenal cell line and the freshwater fish.
- Potential consequences of such mixture toxicity should warrant further investigation.