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DIRECCION

Este trabajo se realizó bajo la dirección del Dr. Leobardo Manuel Gómez Oliván, la Dra. Marcela Galar Martínez y el Dr. Arturo Colín Cruz, en el laboratorio de Toxicología Ambiental, de la Facultad de Química de la Universidad Autónoma del Estado de México, dentro del proyecto CONACYT con clave 151665.

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ABREVIATURAS

AINES	Antiinflamatorios no esteroideos
PTAR	Plantas tratadoras de aguas residuales
DCF	Diclofenaco
IBP	Ibuprofeno
AAS	Ácido acetilsalicílico
NPX	Naproxeno
PAR	Paracetamol
LPX	Lipoperoxidación
SOD	Superóxido dismutasa
CAT	Catalasa
GPx	Glutatión peroxidasa
EUA	Estados Unidos de América
EROs	Especies reactivas de oxígeno
PNEC	Concentración prevista sin efecto (por sus siglas en inglés Predicted no effect concentration)
PEC	Concentración ambiental prevista (por sus siglas en inglés Predicted effect concentration)
RL	Radicales libres
GEN	Ácido gentísico
HDP	Ácido o-hidroxihipúrico
SAL	Metabolitos del AAS
CE_{50}	Concentración efectiva 50
CL_{50}	Concentración letal 50
LOAEL	Nivel más bajo en el que se observan efectos adversos (por sus siglas en inglés lowest observed adverse effect level)
CPC	Concentración de proteínas carboniladas
I	Índice de interacción
MN	Micronúcleos

RESUMEN

Treinta millones de personas en el mundo consumen diariamente AINES, un grupo heterogéneo de fármacos utilizados por sus propiedades analgésicas, antipiréticas y antiinflamatorias, en 2004 fue el sexto grupo farmacológico más importante en volumen de ventas. Recientemente diversos estudios han referido la presencia de grandes concentraciones de este tipo de medicamentos en efluentes de PTAR, en agua superficiales y en menor proporción en aguas subterráneas, agua potable y sedimentos; por otra parte, también se conoce que estos fármacos producen algunos efectos tóxicos en organismos que viven en dichos cuerpos de agua, sin embargo aún no se conoce el efecto que puede producir una mezcla de estos fármacos sobre los organismos, por lo que el propósito de este trabajo fue evaluar la toxicidad producida por el DCF, IBP y la mezcla de IBP-DCF sobre una especie de interés comercial, *Cyprinus carpio*, para esto, se determinó la CL_{50} del IBP y del DCF por separado, estos valores sirvieron para determinar la concentración equivalente al LOAEL de cada fármaco las cuales se utilizaron en el estudio subletal en el que se realizó la evaluación del estrés oxidativo haciendo uso de los siguientes biomarcadores: LPX y la actividad de las enzimas antioxidantes SOD, CAT y GPx, además de la evaluación del daño genotóxico a través de la prueba de micronúcleos y el daño citotóxico mediante la actividad de la caspasa-3. Los resultados muestran que el IBP, DCF y la mezcla IBP-DCF producen estrés oxidativo en sangre, cerebro, branquias e hígado de *Cyprinus carpio*, el mayor daño por lipoperoxidación se presenta a las 24 h en branquias, en cuanto a la actividad de las enzimas el órgano que presenta mayor actividad de la SOD fue el cerebro a las 72 h, para CAT las branquias a las 24 h presentan la mayor actividad mientras que para GPx las branquias a las 48 h presentan el mayor aumento de la actividad. La actividad de la caspasa-3 no presentó diferencia estadísticamente significativa respecto al testigo ($P<0.05$) utilizando la mezcla IBP-DCF, sin embargo el IBP produce aumento de la actividad de esta enzima a las 24 h. En la evaluación del recuento de micronúcleos la mezcla IBP-DCF produce un aumento estadísticamente significativo respecto al testigo ($P<0.05$) a las 24, 48 y 72 h. Los resultados obtenidos muestran que la mezcla IBP-DCF produce interacciones de tipo antagónico con los biomarcadores utilizados, la excepción es la CAT que produce sinergismo en todos los órganos. En conclusión, el IBP, DCF y la mezcla IBP-DCF producen estrés oxidativo, genotoxicidad y citotoxicidad en sangre, cerebro, hígado y branquias de *Cyprinus carpio* y el tipo de interacción toxicodinámica que produce la mezcla IBP-DCF es antagónica en la mayoría de los biomarcadores estudiados.

ABSTRACT

Thirty million people worldwide consume daily NSAIDs, a heterogeneous group of pharmaceuticals used for its analgesic, antipyretic and anti-inflammatory properties, in 2004 were the sixth most important pharmacological group in sales volume. Recently, several studies have reported the presence of large concentrations of these drugs in effluents from wastewater treatment plants (WWTP) in surface water, ground water, drinking water and sediments, on the other hand, is also known these drugs produce some toxic effects on organisms living in these water bodies, but is still unknown the effect that can produce a mixture of these drugs on different aquatic organisms, so the purpose of this study was to evaluate the toxicity produced by diclofenac (DCF), ibuprofen (IBP) and IBP-DCF mixture on a specie of commercial interest, *Cyprinus carpio*, for this, we determined the LC_{50} of IBP and DCF separately, these values were used to determine the equivalent concentration of LOAEL of each drug which was used in the sublethal study, for this it was performed the evaluation of oxidative stress using the following biomarkers: lipidperoxidation (LPX) and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as the evaluation of genotoxic damage by the % micronuclei and cytotoxic damage through the activity of caspase-3. The results show that the IBP, DCF and IBP-DCF mixture cause oxidative stress in the blood, brain, and liver gills *Cyprinus carpio,* most damage by lipid peroxidation occurs at 24 h in the gills, the organ which had the highest increase in SOD activity was the brain at 72 h, for CAT gills at 24 h showed the highest activity while in GPx activity gills at 48 h showed the highest increase. The activity of caspase-3 not show statistically significant difference from the control ($P < 0.05$) using the IBP-DCF mixture, however the IBP increased activity of this enzyme at 24 h. In assessing % micronucleus IBP-DCF mixture produces a statistically significant increase compared to the control ($P < 0.05$) at 24, 48 and 72 h. The results show that IBP-DCF mixture produces antagonic interactions with biomarkers used, the exception is CAT in which synergism interaction occurs in all organs. In conclusion, IBP, DCF and IBP-DCF mixture causes oxidative stress, genotoxicity and cytotoxicity in blood, brain, liver and gills of *Cyprinus carpio* and the type of toxicodynamics interaction that occurs with IBP-DCF mixture is antagonic in most biomarkers studied.

CAPÍTULO 1

“PROTOCOLO DE TESIS”

1. INTRODUCCIÓN

Los AINES son un grupo heterogéneo de fármacos frecuentemente utilizados debido a sus propiedades analgésicas, antiinflamatorias y antipiréticas. Los miembros más comunes por consumo y actividad biológica de este grupo de medicamentos son: el AAS, PAR, DCF, IBP y NPX, entre otros (Katzung, 2007).

Un estudio efectuado por Langman en 1988, estimó que las prescripciones de AINES en EUA superaban los 100 millones anuales, para 1999 uno de cada 7 sujetos en ese país tomaba un AINE diariamente (Bjorkman, 1999). En el Reino Unido se prescriben 24 millones de recetas anuales, un 15% de su población mayor toma AINES y en 2004, fue el sexto grupo farmacológico más importante en volumen de ventas. Actualmente, se refiere que treinta millones de personas en el mundo consumen diariamente AINES (Salas, 2007). En México no se cuenta con datos oficiales proporcionados por la Secretaría de Salud, acerca del consumo, sin embargo se encuentran dentro de los primeros lugares de medicamentos consumidos (Gómez-Oliván et al., 2009; Angeles-Chimal et al., 1992).

Los medicamentos de consumo humano y veterinario se han convertido en una fuente importante de contaminación, denominados contaminantes de preocupación emergente, dado que diversos estudios han demostrado un incrementado notablemente de su concentración en los mantos acuíferos a nivel mundial. Muchos de estos compuestos son considerados tóxicos para los organismos acuáticos, debido a que dependiendo de su naturaleza y concentración, pueden causar daños letales o crónicos en los mismos (Kent et al, 2006; Cleuvers, 2004; Zinder et al., 2004; Montforts et al., 2007).

El uso de biomarcadores de contaminación ha sido sujeto de diversos estudios (Ron van der Oost et al. 2003). Algunos biomarcadores a nivel bioquímico incluyen estudios en los que el objetivo es evaluar el estrés oxidativo a través del daño a macromoléculas inducido por EROs (Barata et al., 2005).

El efecto tóxico de muchos xenobióticos está mediado por la formación de EROs. Un xenobiótico es capaz de inducir estrés oxidativo como resultado de un proceso cíclico de oxidación-reducción en el cual, al ser biotransformado, sufre la reducción por un electrón donado por el NADPH

transformándose luego en un intermediario o especie reactiva que puede ser un radical libre (Abdollahi et al., 2004). Esta especie reactiva, al intentar recuperar su configuración original, busca transferir o donar su electrón no apareado al oxígeno (O_2). así el O_2 se reduce transformándose en el anión superóxido (O_2^-) con la consecuente regeneración del componente parental. El O_2^- reacciona a su vez con otras moléculas o consigo mismo y genera el radical hidroxilo (OH^-) que mediante una serie de reacciones en cadena y su capacidad de oxidar macromoléculas puede ocasionar la muerte celular (Livinsgston, 2001).

Los resultados obtenidos en lo que refiere a la genotoxicidad y citotoxicidad, se consideran indicadores útiles de los efectos de los contaminantes en los ecosistemas acuáticos, porque este tipo de efectos puede estar asociada con trastornos orgánicos que pueden afectar a la fecundidad, y el ciclo biológico del organismo involucrado (Theodorakis et al., 2000). Ambos efectos se han relacionado con un aumento en la producción de EROs (Huang et al., 2007).

El estrés oxidativo es ocasionado por un desbalance entre las EROs y los sistemas antioxidantes del organismo. Las EROs son producidas en las células como resultado de los procesos metabólicos que llevan a cabo (Vlahogianni et al., 2007). Para minimizar el daño oxidativo a los componentes celulares, los organismos han desarrollado defensas antioxidantes. Las enzimas antioxidantes más importantes son la SOD (convierte O_2^- a H_2O_2), CAT (reduce H_2O_2 a agua) y GPX (detoxifica H_2O_2 ó hidroxiperóxidos orgánicos producidos, por ejemplo, por la LPX) (Barata et al., 2005).

Para evaluar el impacto tóxico de los contaminantes presentes en cuerpos de agua se pueden emplear diversos bioindicadores. Los organismos acuáticos se eligen como especie de ensayo debido a su capacidad de filtración, la facilidad de su cultivo y a su sensibilidad al daño oxidativo en exposiciones crónicas o concentraciones subletales. (Livingstone et al., 2003). Los estudios de toxicidad en organismos de teleósteos son necesarios para comprender los mecanismos de acción, para predecir y evaluar la toxicidad, sobre todo si la contaminación se considera crónica. Una especie comúnmente usada como bioindicador es *Cyprinus carpio* (Huang et al, 2007). Esto se debe a que los ciprínidos son cuantitativamente el grupo de peces teleósteos más importantes cultivados con fines comerciales, alrededor del mundo ya que son organismos muy resistentes y

fáciles de mantener (Kestemont, 1995).

Existen diversos estudios que refieren que los AINES como el IBP, DCF, NPX y AAS a bajas concentraciones son capaces de producir estrés oxidativo y daño a las macromoléculas (Oviedo-Gómez et al., 2010; Gómez-Oliván et al., 2012; Islas-Flores et al., 2013). Sin embargo, la toxicidad en los ecosistemas naturales por lo general no resulta de la exposición a una sola sustancia, sino que es resultado de la exposición a mezclas de sustancias tóxicas y son pocos los estudios que evaluan la toxicidad de estas.

Por lo anterior, el objetivo de este trabajo fue determinar la toxicidad producida por el IBP, DCF y la mezcla IBP-DCF usando como bioindicador a *Cyprinus carpio* a través de biomarcadores de estrés oxidativo, genotoxicidad y citotoxicidad e identificar el tipo de interacción toxodinámica que se presenta mediante un análisis isobolográfico.

2. ANTECEDENTES

Medicamentos de venta libre tales como: estimulantes (cafeína), AINES como el IBP, AAS, PAR, y fármacos que requieren prescripción médica como carbamazepina, atorvastatina, fluoxetina y 17β -etinilestradiol, se encuentran presentes en las aguas residuales municipales (Gagné et al., 2005).

Los AINES son fármacos que se han encontrado más frecuentemente en los cuerpos de agua a nivel mundial. Forman parte de los seis fármacos más vendidos en el mundo y usualmente se encuentran en cantidades significativas en los efluentes municipales (Parolini et al., 2009). Esto ha sido comprobado mediante diversos estudios alrededor del mundo, en la Tabla 1 se muestra un resumen de algunos antecedentes de estudios que demuestran la presencia de dichos fármacos en cuerpos de agua.

Tabla 1. Antecedentes de la presencia de AINES en ecosistemas acuáticos.

Autor (Año)	Conclusión
Ferrari et al., (2003)	Midieron la ocurrencia en efluentes de PTAR en Francia, Grecia, Italia y Suiza de los fármacos carbamacepina, ácido clofíbrico y DCF, además hicieron bioensayos en bacterias, algas, microcrutáceos y peces, encontrando que la carbamacepina es el más peligroso, sin embargo el PEC/PNEC del DCF fue de 0.16 y se encontró que fue el fármaco con la mayor concentración en el efluente de la PTAR con una concentración de 5.45 ug/L, además calcularon su EC_{50} en *D. rerio* siendo de 8000 µg/L y para *D. magna* de 22704 µg/L.
Asthon et al., (2004)	Investigaron la incidencia de 12 compuestos farmacéuticos y sus metabolitos en efluentes de PTAR y agua superficial en el Reino Unido. Diez de los doce compuestos farmacéuticos analizados fueron detectados en muestras de PTAR estos fueron: propanolol en concentraciones de 76 ng/L, DCF 424 ng/L, IBP 3086 ng/L, ácido mefenámico 133 ng/L, dextropropoxifeno 195 ng/L, trimetoprim 70 ng/L, eritromicina <10 ng/L, acetilsulfametoxazol <50 ng/L, sulfametoxazol <50 ng/L y tamoxifen <10 ng/L.
Ferrari et al., (2004)	Encontraron que las concentraciones predecibles y las medidas en efluentes de Francia y Alemania exceden los 10 ng/l para los fármacos: carbamacepina, ácido clofíbrico, DCF, ofloxacino, propanolol y sulfametoxazol, además que la toxicidad aguda es baja.
Zuccato et al., (2005)	En efluentes de los ríos Lambro y Po en Italia identificaron ofloxacino, furosemida, atenolol, hidroclorotiazida, carbamacepina, IBP, bezafibrato, eritromicina y claritromicina en concentraciones de 0.1 a 67 ng/L, el IBP se encontró en concentraciones de 13.0 a 20.0 ng/L.

Bendz et al., (2005)	Encontraron que el IBP, ketoprofeno, NPX y DCF son objeto de importantes transformaciones bióticas y abióticas o de secuestro en un río de Suiza.
Lin y Reinhard , (2005)	Estudiaron la fotodegradación en agua purificada y de río con concentraciones de 1-2 µg/L irradiados con una lámpara de xenon, el tiempo de vida media para el ketoprofeno, fue de 4.1 min, 1.1 min para propanolol, 1.4 min para NPX, 15 h para gemfibrozil e iIBP.
Lindqvist et al., (2005)	Reportan que el tratamiento del agua en 7 PTAR de Finlandia para IBP, NPX, ketoprofeno, DCF y bezafibrato. La remoción fue mayor para IBP (92% +/-8%) y menor para el DCF (26%+/-17%), encontraron que en los ríos y puntos de descarga de estas PTAR las concentraciones son más bajas por estar más diluidas y a las concentraciones encontradas en el agua superficial no se encuentran riesgos de toxicidad aguda.
Kosjek et al., (2005)	No hay trazas de AINES (IBP, NPX, ketoprofeno y DCF) en muestras de agua potable, y en ríos de Eslovenia el nivel de contaminación es bajo (ng/L), del DCF se encontraron concentraciones que van de 9 a 282 ng/L.
Stülten et al., (2008)	Analizaron 6 efluentes de PTAR en Alemania y encontraron cantidades de DCF y sus metabolitos: 4'-hidroxidiclofenaco y 5'-hidroxidiclofenaco a concentraciones de 0-0.6 µg/L de DCF y de sus metabolitos de 1.3 – 3.3 µg/L.
Corneau et al., (2008)	Estudiaron la incidencia de fármacos en el efluente de una PTAR en Atlantic, Canadá. El NPX, IBP y la cafeína fueron los más predominantes. El NPX, IBP y el ácido salicílico se detectaron en rangos bajos de ng/l en aguas tratadas y no tratadas.
Siemens et al., (2008)	Encontraron concentraciones de DCF de 0.25-0.50 µg/L y de IBP de 3.5 µg/L en el sistema de irrigación del Valle de Mezquital en México D.F.
Letzel et al., (2009)	Reportan DCF en concentraciones mayores a 2200 ng/L en efluentes de Alemania, a estas concentraciones pueden ocurrir efectos adversos crónicos en las poblaciones de peces.
Yamamoto et al., (2009)	La biodegradación es lenta (> 24 h) y depende del lugar de muestreo y el tiempo. El propanolol, indometacina, ifenpradil son fácilmente fotodegradados.
Marco-Urrea et al.,(2009)	El IBP es degradado a 1-hidroxibuprofeno y 2-hidroxibuprofeno por los hongos *Trametes versicolor, Irpex lacteus*, *Phonerochaete chrysosporium* y *Ganoderma lucidum.*
Ferguson et al., (2013)	Reportan concentraciones de 5.36 ng/L de PAR, 7.88 ng/L de IBP, 6.32 ng/L de NPX en el lago Michigan (EUA), las concentraciones no difieren con el sitio o la profundidad, sin embargo, la temperatura, el carbono total, los sólidos disueltos, el oxígeno total y la concentración de amonio están relacionados con las concentraciones totales de los farmacéuticos.

El desarrollo de técnicas analíticas más sensibles ha dado lugar a una mayor conciencia de la presencia de los productos farmacéuticos en el medio ambiente, la preocupación ahora se está centrando en cuanto a los posibles efectos adversos que estos compuestos pueden tener sobre los

organismos que habitan estos ecosistemas, especialmente en condiciones de exposición crónica (Madden et al., 2009). Los fármacos son abundantes a concentraciones de ng/L a g/L en sistemas acuáticos naturales (Schreiber et al., 2008), es por esta razón que existen diversos estudios realizados sobre la presencia de AINES en medios acuáticos y los efectos tóxicos que provocan sobre las especies que ahí habitan, en la Tabla 2 se presentan algunos antecedentes de este tipo de estudios.

Tabla 2. Antecedentes del efecto de AINES sobre organismos acuáticos.

Autor (Año)	Medicamento	Conclusión
Stuer-Lauridsen et al., (2000)	25 fármacos más utilizados	De 25 fármacos más utilizados y encontrados en efluentes obtuvieron el PEC y el PNEC, encontraron que la relación PEC/PNEC para el IBP, el AAS y PAR es mayor a 1.
Marques et al., (2004a)	GEN, HDP, otros metabolitos del AAS (SAL).	Evaluaron la toxicidad aguda y crónica en *D. magna y D. longispira,* encontrando que la toxicidad del GEN>SAL>HDP, el HDP no presenta toxicidad aguda, pero en exposición crónica produce neonatos anormales y abortos. El SAL y GEN producen cambios en la reproducción y crecimiento. *D. longispira* es la más sensible a estos fármacos.
Richards et al., (2004)	IBP, fluoxentina, ciprofloxacino	Hay poca respuesta a concentraciones de 6, 10 y 10 µg/L (concentración baja) respectivamente, a concentraciones de 60, 100 y 100 µg/L (concentración media) y de 600, 1000 y 1000 µg/L (concentración alta) se produce muerte de peces.
Marques et al., (2004b)	AAS	Estudiaron la toxicidad crónica en cladóceros, encontrando que afecta la reproducción a concentraciones de 1.8 mg/L.
Cleuvers, (2004)	DCF, IBP, NPX, AAS	Encontraron que la toxicidad es relativamente baja. La $CE_{50_{50}}$ es 68-166 mg en Daphnidos y 72-626 mg en algas. Entre mayor es el log Kow mayor es la toxicidad. La toxicidad de mezclas de estos compuestos pueden predecirse usando el concepto de adición de concentración. La toxicidad de la mezcla fue considerable incluso a concentraciones que las sustancias simples no muestran efectos.
Yamamoto et al., (2005)	IBP, PAR, atenolol, fluoxentina	Determinaron el coeficiente de sorción de cada fármaco, encontrando que el de fluoxentina es mayor que el del atenolol y del IBP.

Isidori et al., (2005)	NPX	Los fotoproductos son más tóxicos que el compuesto original en pruebas agudas y crónicas. No se encontraron efectos genotóxicos o mutagénicos en algas, rotíferos y microcrustáceos.
Richards et al., (2006)	Inhibidores selectivos de serotonina, reguladores de lípidos en sangre, AINES, antibióticos, un estimulante y un antiepiléptico.	Expusieron *Xenopus* durante 96 h a una concentración comúnmente prescrita de cada fármaco. Se determinó la toxicidad, teratogenicidad, concentración mínima para inhibir el crecimiento, tipos y severidades de malformaciones y se encontró que la toxicidad varia entre las clases de fármacos analizados, así los fluoroquinolonas, cafeina, PAR, carbamazepina y antibióticos son reportados no teratogénicos a exposiciones de 100 mg/L durante 96 h.
Brun et al., (2006)	PAR, IBP, NPX, Acido salicílico	No encontró ningún efecto negativo en concentraciones de 32 a 500 µg/L en la evaluación de la toxicidad aguda para *Daphnia magna* y *Vibrio fischeri* respectivamente. En cuanto a la toxicidad crónica únicamente se aprecio en la alga *Selanastrum capricornutum* con el IBP.
Gravel et al., (2007b)	AAS	Reportan que no hay cambios significativos en la hexocinasa del hígado , glucocinasa, lactato deshidrogenasa, piruvato cinasa, fosfoenolpiruvato carboxilasa, aspartato aminotransferasa y alanina amino transferasa de *Oncorhynchus mykiss.*
Felis et al., (2007)	IBP	Encontraron que la CE_{50} en *D. magna* es 10-100 mg/L en 14 días disminuye el crecimiento y la supervivencia se afecta a 80 mg/L. La reproducción se afecta a 13.4 mg/L y se inhibe completamente a 80 mg/L.
Flipin et al., (2007)	IBP	Una mayor exposición a IBP provoca aumento del número de huevos de *Oryzias latipes* por evento reproductivo, pero disminuye el número de desoves por semana La exposición crónica puede modificar el patrón de reproducción.
Kim et al., (2007)	PAR Carbamazepina Cimetidina Diltiazem Sulfonamida	*D. magna* es más susceptible que *D.longispira*. El diltiazem es el más tóxico (CL 8.2 mg/L en daphnidos) La concentración de los fármacos va de 0.14 – 16.5 µg/L en el medio ambiente.
Khetan y Collins, (2007)	AAS	Estudiaron la toxicidad aguda en *D. magna,* a 48 horas la CE_{50} es de 88.1 mg/L, también realizaron la prueba de inhibición de crecimiento con el alga verde *D. Subspicatus* a 3 días reportando que la CE_{50} es de 106.7 mg/L.

Hayashi et al., (2008)	IBP	Realizaron una exposición de 10 días en *D. magna,* encontraron que en la mayor concentración la descendencia es menor, dando 10 días de recuperación la descendencia es igual al control, ya que se recuperan teniendo descendencia más rápido.
Zhang et al., (2008)	Carbamacepina, DCF	Estudiaron la ocurrencia de ambos fármacos en varios cuerpos de agua que incluían efluentes de PTAR y agua purificada, encontraron que no causan efectos tóxicos agudos a las concentraciones ambientales, sin embargo debe tener importancia los efectos crónicos.
Quinn et al., (2008)	IBP, NPX, gemfibrozil, bezafibrato, carbamazepina, sulfametoxazol, sulfapiridina, oxitetraciclina, novobiocina y trimetoprim	Realizaron un ensayo de regeneración de *Hydra attenuata* para conocer el potencial teratogénico de 10 fármacos identificados de un efluente de una PTAR. Se inhibió la regeneración con concentraciones de 1, 5 y 1 mg/L de gemfibrozil, IBP y NPX, y concentraciones más altas de 50 mg/L para bezafibrato y trimetropin. Carbamacepina y antibioticos (sulfapiridina, oxitetraciclina y novobicin) incrementan la regeneración a 25,5,50 y 50 mg/L respectivamente. Tienen potencial teratogénico: Gemfibrozil, IBP, NPX y bezafibrato. El que tiene menor protencial teratogénico es la carbamacepina
Parolini et al., (2009)	DCF, IBP, PAR	Encontraron que el potencial de citogenotoxicidad es mayor para el PAR, le sigue el DCF y por último el IBP usando biomarcadores in vitro (ensayo cometa y ensayo de retención de rojo neutro) en hemocitos de *Dreissena polymorpha.*
Quin el al., (2009)	IBP, NPX, gemfifrozil, bezafibrato, carbamacepina, sulfapiridina, oxitetraciclina, novobiocin, trimetropin, sulfametoxazol	Los fármacos actúan aditivamente en una mezcla, con efectos subletales a concentraciones relevantes en el medio ambiente (ng/L a ⌠g/L).

Oviedo-Gómez et al., (2010)	DCF	Determinaron la toxicidad aguda y subletal sobre *Hyalella azteca* utilizando biomarcadores de estrés oxidativo (LPX, CPC, actividad de SOD, CAT y GPx). La CL50-72 h fue de 0.467 mg/kg, y los resultados del estudio subletal mostraron un incremento significativo a todos los tiempos de exposición del grado de LPX y CPC, además de un cambio en la actividad de las enzimas SOD, CAT y GPx.
Gómez-Oliván et al., (2012)	PAR	Evaluaron la exposición subletal utilizando biomarcadores de estrés oxidativo (LPX, CPC, actividad de SOD, CAT y GPx), reportaron un incremento estadísticamente significativo con respecto al testigo en el grado de LPX y CPC, la actividad de las enzimas SOD, CAT y GPx disminuyó.

3. JUSTIFICACIÓN

En México, como en muchos países del mundo, las principales fuentes de contaminación del agua se clasifican en tres grupos, de acuerdo con su procedencia: el sector social (comprende las descargas domésticas y públicas), el sector agropecuario y el sector industrial. En esta agua residual se encuentran farmacéuticos en concentraciones traza provenientes de los tres sectores, los cuales pueden no ser eliminados por los procesos de tratamiento y llegar nuevamente a los cuerpos de agua y a los sedimentos en donde se encontrarán en contacto con los hidrobiontes, organismos micro y macroinvertebrados, algas y peces, los cuales nos sirven como bioindicadores del efecto tóxico que pueden producir estos farmacéuticos a través de biomarcadores de exposición, suceptibilidad y efecto. A pesar de que hace varios años se conoce la presencia de los farmacéuticos y productos para el cuidado personal en los cuerpos de agua y de sus efectos tóxicos sobre la flora y la fauna de estos ecosistemas, en nuestro país no existen muchos estudios al respecto, por lo que este estudio será una aportación a esta área del conocimiento. El objetivo de este trabajo es evaluar el efecto toxicológico del IBP, DCF y la mezcla IBP-DCF, dos fármacos que pertenecen a la clase de los antiinflamatorios no esteroideos, fármacos de venta libre que son altamente consumidos por sus propiedades antiinflamatorias, antipiréticas y analgésicas, utilizando como bioindicador una especie que igualmente es altamente consumida y de interés comercial, *Cyprinus carpio*. El efecto toxicológico se evaluará calculando la CL_{50} y posteriormente utilizando biomarcadores de efecto (estrés oxidativo, daño citotóxico y genotóxico) para conocer como es que actúan estos fármacos en una mezcla y utilizando el análisis isobolográfico reportar el tipo de interacción toxodinámica que generan.

4. HIPÓTESIS

Si se adiciona IBP, DCF y la mezcla IBP-DCF a agua sintética en concentraciones traza se producirá estrés oxidativo, citotoxicidad y genotoxicidad en carpas, lo que modificará los niveles de oxidación de lípidos, además de la actividad de las enzimas antioxidantes SOD, CAT y GPx, así como la actividad de la caspasa 3, el % de micronúcleos en sangre, branquias, hígado y cerebro de *Cyprinus carpio* .

5.OBJETIVOS

5.1 Objetivo general

Evaluar la toxicidad producida por el IBP, DCF y la mezcla IBP-DCF empleando *Cyprinus carpio.*

5.2 Objetivos específicos

1. Determinar la toxicidad aguda del IBP, DCF y la mezcla IBP-DCF empleando como bioindicador a *Cyprinus carpio.*

2. Determinar el estrés oxidativo del IBP, DCF y la mezcla IBP-DCF en sangre, hígado, cerebro y branquias de *Cyprinus carpio*, a través de los siguientes biomarcadores: grado de lipoperoxidación y la actividad de las enzimas superóxido dismutasa, catalasa y glutatión peroxidasa.

3. Determinar el daño genotóxico del IBP, DCF y la mezcla IBP-DCF en sangre de *Cyprinus carpio* a través de la prueba de micronúcleos.

4. Determinar el daño citotóxico del IBP, DCF y la mezcla IBP-DCF a través de la actividad específica de la caspasa-3 en sangre de *Cyprinus carpio*.

5. Determinar el tipo de interacción toxicodinámica que produce la mezcla IBP-DCF a través de un análisis isobolográfico.

6. METODOLOGÍA

6.1 Obtención y aclimatación del organismo de prueba

Se utilizaron ejemplares de carpa común, *Cyprinus carpio*, las cuales se obtuvieron del centro acuícola Tiacaque, Estado de México. Los organismos se mantuvieron en acuarios de 120 L de capacidad durante un período de adaptación de 15 días, a temperatura ambiente con periodos de luz oscuridad natural y alimentadas con Purina^{MR} para peces diariamente.

6.2 Preparación del agua reconstituida

Se disolvió en 2.85 L de agua destilada o desionizada, 120 mg/L de $MgSO_4$, 174 mg/L de $NaHCO_3$, 8 mg/L KCl en el orden descrito. Por otra parte se agregó 120 mg/L de $CaSO_4 \cdot 2H_2O$ a 150 mL de agua destilada o desionizada en un matraz erlenmeyer de 250 mL. Se colocó la solución sobre un agitador magnético hasta que el sulfato de calcio estuvo completamente disuelto. Se adicionó a los 2.85 L preparados con anterioridad y se mezclo perfectamente.

6.2.1 Caracterización fisicoquímica del agua reconstituida

El agua sintética se caracterizó por medio de la determinación del pH que se medió usando un potenciómetro de electrodo de vidrio (JENCO VisionPlus modelo pH6175), el oxígeno disuelto (usando un oxímetro Simpson-Electric Co. YST Modelo 51-B), la conductividad (Phmetro Conductronic Modelo TPM-PH_{130}).

6.3 Determinación de la toxicidad aguda

Los sistemas de intoxicación consistieron en 45 recipientes de polietileno de 10 L. Se adicionaron cinco concentraciones IBP y DCF (por separado) en agua reconstituida y un testigo que no contenía estos fármacos, por triplicado. Se colocaron 6 organismos (*Cyprinus carpio*) en cada sistema y se observaron los sistemas durante 96 horas, se determinó el número de organismos muertos al finalizar el periodo del estudio. La CL_{50} del IBP y DCF en *Cyprinus carpio* se

determinó a las 96 horas (CL_{50} 96 horas) y se calculó por análisis computarizado de Probits (Método de unidades probabilísticas).

6.4 Estudio subletal y preparación de las muestras

Tres réplicas por grupo de 6 organismos se colocaron en recipientes de polietileno de 10 L de capacidad que contenían 6 L de agua reconstituida sembrada con 1/10 CL_{50} del ibuprofeno, del diclofenaco y de la mezcla ibuprofeno-diclofenaco, además de un sistema libre de fármacos. El tiempo de exposición fue de 12, 24, 48, 72 y 96 h; al finalizar cada tiempo de exposición, los organismos fueron retirados de los sistemas y se colocaron en un recipiente que contenía 50 mg/L de eugenol, con el fin de anestesiar a los organismos (Yamanaka et al. 2011). Se obtuvieron muestras sanguíneas, de branquias, hígado y cerebro. La muestras sanguíneas fueron tomadas en la vena caudal con una jeringa hipodérmica de 1 mL previamente heparinizada y por cada 100 µL se sangre se le adicionaron 400 µL de buffer salino de fosfatos (PBS) pH 7.4, para la obtención de las muestras restantes se realizó una disección en un baño de hielo, después se pesaron y homogenizaron en 2 mL de PBS pH 7.4 y se centrifugaron a 12 500 rpm a -4°C durante 15 min para la obtención del sobrenadante . El sobrenadante se empleó para el análisis bioquímico.

6.4.1 Determinación del grado de lipoperoxidación (Método de Buege y Aust, 1979)

A 500 µL del homogenizado (sin centrifugar) se le adicionaron 1 mL de la solución reguladora tris-HCl (Sigma-Aldrich) 150 mM a pH 7.4. Se incubó a 37°C por 30 min. Después de la incubación se agregaron 2 mL de la solución de ácido tiobarbitúrico (Sigma-Aldrich) al 0.375% (preparada al momento) en ácido tricloroacético (Sigma-Aldrich) al 15% y se agitó en vortex, posteriormente se calentó a ebullición durante 45 min., se dejo enfriar y se removió el precipitado por centrifugación a 3000 rpm por 10 min. Concluido el tiempo se determinó la absorbancia a 535 nm de las muestras contra un blanco de reactivo. Los resultados se expresaron en mM de malondialdehído /mg proteínas/g tejido usando el coeficiente de extinción molecular (CEM) el cual es de $1.56 \times 10^{5}\ M^{-1}cm^{-1}$.

6.4.2 Determinación de la actividad de la superóxido dismutasa (Método de Misra y Fridovich, 1972)

Se colocaron 40 µL del homogenizado en una celda de cuarzo y se adicionaron 260 µL de solución amortiguadora de carbonatos [carbonato de sodio (Sigma-Aldrich) 50 mM y EDTA (Vetec) 0.1 mM] a pH 10.2. Posteriormente, se agregaron 200 µL de adrenalina (Bayer) 30 mM y se determinó la absorbancia a 480 nm a los 30 s y 5 min. La actividad enzimática se determinó usando el CEM de SOD (21 M/m). Los resultados se expresaron como M SOD/mg proteína.

6.4.3 Determinación de la actividad de la catalasa (Método de Radi et al, 1991)

A 20 µL del sobrenadante del homogenizado se le agregaron 1 mL de la solución amortiguadora de aislamiento (0.3 M sucrosa (Vetec), 1 mM EDTA (Vetec), 5 mM HEPES (Sigma-Aldrich) y 5 mM KH_2PO_4 (Vetec)) y 0.2 mL de la solución de H_2O_2 (Vetec) 20 mM. Posteriormente se determinó la absorbancia a 240 nm, a 0 y 60 seg. Los resultados se obtuvieron sustituyendo la absorbancia de ambos tiempos en la siguiente formula: concentración de catalasa = $(A_0 - A_{60})$/ CEM, donde el CEM del H_2O_2 = 0.093 mM-1 cm-1; los datos se expresaron en mM de H_2O_2/min/g tejido.

6.4.4 Determinación de glutatión peroxidasa (Gunzlery Flohe, 1985; modificado por Stephensen et al., 2000)

Se colocaron 100 µL del sobrenadante en una celda de cuarzo y se le adicionaron 10 µL de glutatión reductasa [2 U glutatión reductasa, (Sigma-Aldrich)], además de 290 µL del buffer de reacción [K_2HPO_4 (Vetec) 50 mM, KH_2PO_4 (Vetec) 50 mM pH 7.0, glutatión reducido (Sigma-Aldrich) 3.5 mM, azida de sodio (Sigma-Aldrich) 1 mM, NADPH (Sigma-Aldrich) 0.12 mM)] y 100 µL de H_2O_2 (Vetec) 0.8 mM. Posteriormente se determinó la absorbancia a 340 nm, a los 0 y 60 s. Los resultados se obtuvieron usando la siguiente ecuación: concentración de GPx = $[(A_0 - A_{60})/CEM]$, donde el CEM del NADPH es 6.2 mM/cm. Los resultados se expresaron como mM NADPH/mg proteína.

6.4.5 Determinación de la concentración de proteínas (Método de Bradford, 1976)

A 25 µL del sobrenadante se le agregaron 75 µL de agua desionizada y 2.5 mL del reactivo de Bradford (0.05 g de azul de commassine, 25 mL de etanol al 96% y 50 mL de H_3PO_4 en 500 mL de agua desionizada). Se agitaron los tubos, se dejaron reposar por 5 min y a continuación se determinó la absorbancia a 595 nm. Para la curva tipo se utilizó albúmina bovina (1 mg/mL).

Tabla 3. Curva tipo para la prueba de proteínas totales.

Tubo	Albúmina (µL)	Agua desionizada (µL)
1	10	90
2	25	75
3	50	50
4	75	25
5	100	0
Blanco	0	100

6.4.6 Análisis estadístico

Los datos se analizaron por análisis de varianza unifactorial (ANOVA) y las diferencias significativas de cada grupo se compararon empleando la prueba de Tukey para comparaciones múltiples contra un testigo. Las diferencias fueron consideradas significativas a una $P < 0.05$.

6.5 Evaluación de la genotoxicidad y citotoxicidad

Para el estudio de citotoxidad, se emplearon recipientes de polietileno de 10 L de capacidad, que contenían 1/10 de la CL_{50} del IBP, del DCF y de la mezcla IBP-DCF, además de un sistema libre de fármacos. En cada sistema de prueba (incluyendo al testigo) se colocaron seis organismos. El tiempo de exposición fue de 24, 48, 72 y 96 horas; después del cual, se colocaron en una pecera que contenía 50 mg/L de eugenol (Yamanaka et al. 2011). Posteriormente, se tomó una muestra sanguínea de la vena caudal; por cada 100 µL de sangre se adicionaron 400 µL de

buffer salino de fosfatos (PBS). Con las muestras de sangre de cada individuo se realizaron pruebas para determinar genotoxicidad (% de micronúcleos) y citotoxicidad (actividad de la caspasa-3). El ensayo se realizó por triplicado.

6.5.1 Micronúcleos (Countrymam y Heddle, 1976)

Los MN, es una técnica citogenética que permite detectar el daño en el ADN, así como procesos de reparación fallidos en el ADN de células post-mitóticas. El primer paso para esta técnica fue obtener un frotis de la muestra sanguínea, después se fijó con etanol puro durante 20 min. Posteriormente se tiñó con una solución de Giemsa al 10% durante 9 min. Se observaron en un microscopio de luz un total de 1000 eritrocitos o hemocitos por muestra, la frecuencia de micronúcleos en las células (MN) se expresó como número por mil (%) MN. Losn criterios para determinar la presencia de micronúcleos fueron: no conexión de pequeños núcleos de forma circular u ovoide con el núcleo principal y el área aproximada de una tercera parte del núcleo principal. Las células que presentaron las características anteriores y que mostraron el mismo patrón de tinción se consideraron como micronúcleos.

6.5.2 Actividad de la caspasa-3

Para la determinación de la actividad de caspasa-3 se utilizó un kit de ensayo colorimétrico (CaspACE^{TM} Promega) cuyo sustrato se une a la enzima liberando p-nitroanilina (pNA).

Se preparo un blanco con 32 µL del amortiguador de caspasas, 2 µL de DMSO, 10 µL DTT y 54 µL de agua desionizada; para el grupo testigo y el grupo tratado con los fármacos y las mezclas se utilizaron 32 µL de amortiguador de caspasas, 2 µL de DMSO, 10 µL DDT, 20 µL de extracto celular y 34 µL de agua desionizada; el testigo positivo utilizo 32 µL de amortiguador de caspasas, 2 µL de DMSO, 10 µL DDT, 20 µL de extracto celular y 34 µL de agua desionizada; para la apoptosis inhibida se utilizo 32 µL de amortiguador de caspasas, 2 µL de DMSO, 10 µL DDT, 20 µL de extracto celular con apoptosis inhibida y 34 µL de agua desionizada.

6.5.3 Análisis estadístico

Los datos se analizaron por análisis de varianza unifactorial (ANOVA) y las diferencias

significativas de cada grupo se compararon empleando la prueba de Tukey para comparaciones múltiples contra un testigo. Las diferencias fueron consideradas significativas a una $P < 0.05$.

6.6 Análisis isobolográfico

Este estudio se realizó para la conocer el tipo de interacción de la mezcla IBP-DCF en cada biomarcador evaluado (Miranda *et al.,* 2004). Para esto se utilizó el método modificado de Gessner (1995). Este método plantea la siguiente ecuación:

$$I = dX/dA + dX/dB = 1.0$$

Donde: I es el valor de la interacción, dX es el efecto del biomarcador evaluado en la mezcla binaria del AINE 1 (IBP) y AINE 2 (DCF), y dA y dB son los efectos de los biomarcadores evaluados en los sistemas aislados para el AINE 1 y AINE 2 respectivamente.

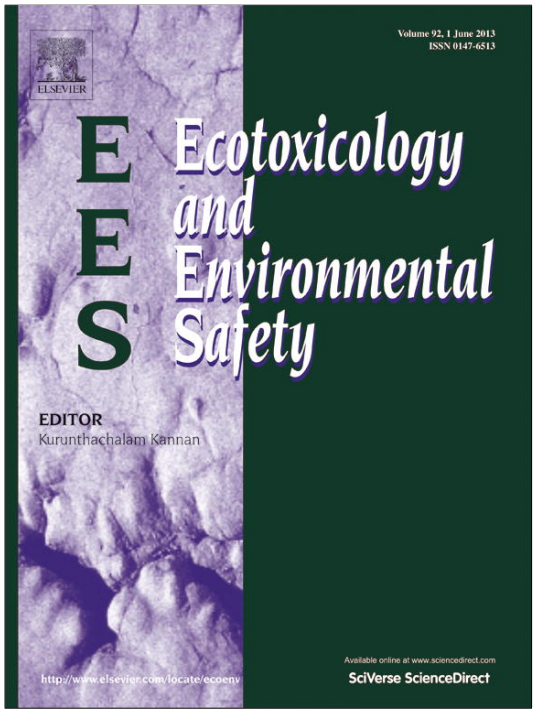
Esta ecuación fue aplicada a los resultados obtenidos de cada biomarcador de estrés oxidativo, genotoxicidad y citotoxicidad evaluado a las 96 h y se consideró que se trataba de una interacción toxodinámica de tipo aditivo, sinérgico o antagónico, según los siguientes resultados:

$$I = dX/dA + dX/dB = 1.0 \text{ (aditividad)}$$
$$I = dX/dA + dX/dB > 1.0 \text{ (antagonismo)}$$
$$I = dX/dA + dX/dB < 1.0 \text{ (sinergismo)}$$

CAPÍTULO 2

“DISCUSIÓN DE RESULTADOS”

7. ARTÍCULOS



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Diclofenac-induced oxidative stress in brain, liver, gill and blood of common carp (*Cyprinus carpio*)

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ABSTRACT

Due to its analgesic properties, diclofenac (DCF) is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs). While residue from this pharmaceutical agent has been found in diverse water bodies in various countries, there is not enough information of its potential toxicity on aquatic organisms, particularly in species which are economically valuable due to their high consumption by humans, such as the common carp *Cyprinus carpio*. This study aimed to evaluate potential DCF-induced oxidative stress in brain, liver, gill and blood of *C. carpio*. The median lethal concentration of DCF at 96 h (96-h LC_{50}) was determined and used to establish the concentration equivalent to the lowest observed adverse effect level (LOAEL). Carp specimens were exposed to this concentration for different exposure times (12, 24, 48, 72 and 96 h) and the following biomarkers were evaluated: lipid peroxidation (LPX) and the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Also, the DCF and 4-hydroxy DCF was determined by LC-MS/MS. Results show a statistically significant LPX increase ($P<0.05$) in liver and gill mainly as well as significant changes in the activity of the antioxidant enzymes evaluated in these organs, with respect to controls ($P<0.05$). The DCF concentrations decreased in water system and increased in the carp. The DCF biotransformation to 4-hydroxy DCF was observed to 12 h. The pharmaceutical agent DCF is concluded to induce oxidative stress on the common carp *C. carpio*, with the highest incidence of oxidative damage occurring in liver and gill. Furthermore, the biomarkers employed in this study are useful in the assessment of the environmental impact of this agent on aquatic species.

1. Introduction

In recent decades, international environmental regulations have focused on the risk posed by emergent contaminants released into the aquatic environment and this issue has become increasingly important. Richardson et al. (2007) define emergent contaminants as nonregulated unstudied compounds posing a risk to aquatic ecosystems, such as drug abuse substances, personal care products, steroids, hormones, surfactants, industrial additives, and pharmaceutical agents.

Many of these compounds are considered toxic to aquatic organisms, since depending on their nature and concentration they may induce chronic or lethal damage on these organisms (Kent et al., 2006). These contaminants include non-steroidal anti-inflammatory drugs (NSAIDs), a heterogeneous group of pharmaceuticals that includes acetylsalicylic acid (ASA), paracetamol, diclofenac (DCF), ibuprofen and naproxen, all of them widely used due to their analgesic, antipyretic and anti-inflammatory properties.

A study by Langman (1988) estimated that the number of NSAID prescriptions written in the US each year topped 100 million. By 1999 one of every seven individuals in that country used NSAIDs daily. In the United Kingdom, 24 million prescriptions for NSAIDs are written each year and 15% of the older population uses NSAIDs, which were the sixth most important group of pharmaceutical agents by sales volume in 2004. Currently, 30 million persons worldwide are said to use NSAIDs on a daily basis (Salas et al., 2007).

In Mexico, while official data from the Ministry of Health are not available regarding the use of NSAIDs, they are among the top pharmaceutical agents used (Gómez-Oliván et al., 2009).

DCF has been detected in significant amounts in municipal effluent. Ternes et al. (1999) report its presence at concentrations

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above 1 μg/L in treatment plant water and lower concentrations in surface water. Other studies have reported concentrations between 10 and 2200 ng/L in effluent from wastewater treatment plants in diverse European countries (Stülten et al., 2008; Letzel et al., 2009).

In Mexico, there are no reports of the presence of contaminants of this type in water bodies apart from a study by Siemens et al. (2008), who found DCF at concentrations of 0.25–0.50 μg/L in the Mexico City-Mezquital Valley irrigation system.

Diverse studies report that trace concentrations of DCF may induce toxic effects on different aquatic organisms as well as developmental, reproductive and renal damage (Marques et al., 2004; Brun et al., 2006).

Some studies have described the effect of DCF on common carp. Saravanan et al. (2011) concluded that different concentrations (1, 10 and 100 μg/L) of DCF had a profound influence on the hematological, biochemical, ionoregulatory and enzymological profiles of the freshwater fish *Cyprinus carpio*. On the other hand, Stepanova et al. (2012), mentioned that the subchronic exposure of early life stages of carp to DCF had effects on mortality and some parameters of oxidative stress (GST, GR and TBARS).

One way to evaluate the damage induced by emergent contaminants present in water bodies is the use of biomarkers, which are defined as any measurable biochemical, physiological or morphologic changes associated with exposure to a toxic agent. Some biomarkers include assays at the biochemical level to evaluate oxidative stress in terms of the damage induced by reactive oxygen species (ROS) on macromolecules. Oxidative stress is produced by an imbalance of ROS and antioxidant systems in the body (Barata et al., 2005). The toxic effects of many xenobiotics are mediated by ROS formation as a result of redox cycling (Abdollahi et al., 2004). The antioxidant enzymes that organisms use to offset oxidative damage include superoxide dismutase (SOD), which converts $O_2^{\cdot}$ to H_2O_2; catalase (CAT), which reduces H_2O_2 to water; and glutathione peroxidase (GPx) which detoxifies H_2O_2 or organic hydroperoxides to water (Barata et al., 2005).

Organisms are able to adapt to increased ROS production by increasing regulation by antioxidant defenses (Livingstone, 2003). The failure of antioxidant defenses to detoxify excess ROS production may lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (LPX) (Halliwell and Gutteridge, 1999). The latter in particular is considered the primary mechanism through which oxyradicals can potentially damage tissues, hampering cell function and inducing alterations in the physicochemical properties of the cell membrane leading to disruption of vital functions (Rikans and Hornbrook, 1997).

The balance between prooxidant endogenous and exogenous factors (environmental pollutants) and antioxidant defenses (enzymatic and nonenzymatic) in biological systems can be used to assess toxic effects under stressful environmental conditions, especially oxidative damage induced by different classes of chemical pollutants. The oxidative damage to lipids, DNA, and proteins and the adverse effects on the antioxidant, enzymatic defense mechanisms of aerobic organisms have been used in recent years as biomarkers for monitoring environmental pollution (Valavanidis et al., 2006). The most important stress oxidative biomarkers used in toxicological studies in aquatic systems are lipid peroxidation level, lipid hydroperoxides content, protein oxidation biomarkers and enzymatic antioxidant defenses (Dröge, 2003).

The common carp *C. carpio* is commonly used as a bioindicator species (Huang et al., 2007) since cyprinids are quantitatively the most important group of teleost fishes cultured throughout the world for commercial purposes and are also very resistant, easy to maintain organisms.

Toxicity assays can be performed under simulated conditions in which the contaminant is added to (water or artificial sediment) systems in order to determine the specific effect of the test substance, or else by using matrices containing water or sediment from natural water bodies. The latter procedure permits determination of the toxicity induced by a mixture of contaminants present in the water body as well as potential interactions between xenobiotics and matrix components.

The aim of this study was to evaluate the toxicity induced by diclofenac in a sub lethal concentration on various organs and tissues of the freshwater teleost fish *C. carpio*, using biomarkers of oxidative stress to assess the potential risk posed by water-borne pharmaceuticals to the physiology and survival of aquatic organisms.

2. Materials and Methods

2.1. Test substance

The pharmaceutical agent (CAS number 15307-86-5, >99% purity) $C_{14}H_{11}Cl_2NO_2$, 296 Da, was purchased from Sigma-Aldrich. Stock solutions were prepared by dissolving 1 g DCF in deionized water.

4-hydroxy DCF (4-OH DCF) agent (CAS number 64118-84-9, >98% purity HPLC) $C_{14}H_{11}Cl_2NO_3$, 312.15 Da, was purchased from Sigma-Aldrich.

2.2. Specimen collection and maintenance

Common carp (*C. carpio*) 18.39 ± 0.31 cm in length and weighing 50.71 ± 7.8 g were obtained from the aquaculture facility in Tiacaque, State of Mexico. Fish were safely transported to the laboratory in well-sealed polyethylene bags containing oxygenated water and subsequently stocked in a large tank with dechlorinated tap water (subsequently reconstituted with salts) and acclimated to test conditions for 30 days prior to the experiment. During acclimation, carp were fed Pedregal Silver™ fish food, and three-fourths of the tank water was replaced every 24 h in order to maintain a healthy environment. The physicochemical characteristics of tap water reconstituted with salts were maintained, i.e. temperature 20 ± 2 °C, oxygen concentration 80–90%, pH 7.5–8.0, total alkalinity 17.8 ± 7.3 mg/L, total hardness 18.7 ± 0.6 mg/L. A natural light/dark photoperiod was maintained.

2.3. Median lethal concentration (LC_{50}) and oxidative stress determination

Test systems consisting in 120 × 80 × 40-cm glass tanks filled with water reconstituted from the following salts: $NaHCO_3$ (174 mg/L, Sigma-Aldrich), $MgSO_4$ (120 mg/L, Sigma-Aldrich), KCl (8 mg/L, Vetec) and $CaSO_4 \cdot 2H_2O$ (120 mg/L, Sigma-Aldrich) were maintained at room temperature with a natural light/dark photoperiod and provided with constant aeration. Static systems were used and no food was provided to specimens during the exposure period.

To establish the target concentration to be used for oxidative stress evaluation, the median lethal concentration (LC_{50}) of DCF was determined. To this end, six test systems to which were added different nominal concentrations of DCF (9.5, 18.9, 37.7, 75.2, 300.8, 600 mg/L) and a seventh DCF-free control system were set up, and 10 carp randomly selected from the stock (using the random number method) were placed in each system. In total 350 fish were used in the median lethal concentration determination.

Duration of the exposure period was 96 h, at the end of which the number of dead specimens in each system was counted. The assay was performed in quintuplicate. The 96-h LC_{50} of DCF and its 95% confidence limits ($P<0.05$) were estimated by Probit analysis (EPA, v1.5). The data obtained were used to estimate the concentration to be used in the assays for oxidative stress determination.

Sublethal toxicity assays involved adding DCF at a concentration equivalent to the lowest observed adverse effect level (LOAEL), i.e. 7.098 mg/L, to five test systems with 6 carp each. A kinetics was run for the following exposure periods: 12, 24, 48, 72 and 96 h. DCF-free control system were set up for each exposure time in the sub lethal study.

A DCF-free control system with 6 carp was set up for each exposure period, and sublethal assays were performed in triplicate. In total 180 fish were used in the sublethal assays. At the end of the exposure period, fish were removed from the systems and placed in a tank containing 50 mg/L of clove oil as an anesthetic (Yamanaka et al., 2011). Anesthetized specimens were placed in a lateral position and blood was removed by puncture of the caudal vessel using a heparinized 1-mL hypodermic syringe, and performed laterally near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line.

After puncture, specimens were placed in an ice bath and sacrificed. The gill, liver and brain were removed, placed in phosphate buffer solution pH 7.4 and homogenized. The supernatant was centrifuged at 12,500 rpm and −4 °C for 15 min. The

following biomarkers were then evaluated: LPX and the activity of the antioxidant enzymes SOD, CAT and GPx. All bioassays were performed on the supernatant.

2.3.1. Determination of LPX

LPX was determined by the Büege and Aust (1978) method. To 100 mL of supernatant was added Tris–HCl buffer solution pH 7.4 (Sigma-Aldrich) until a 1 mL volume was attained. Samples were incubated at 37 °C for 30 min; 2 mL TBA–TCA reagent [0.375% thiobarbituric acid (TBA, Sigma-Aldrich) in 15% trichloroacetic acid (TCA, Sigma-Aldrich)] was added and samples were shaken in a vortex. They were then heated to boiling for 45 min, allowed to cool, and the precipitate removed by centrifugation at 3000 rpm for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56×10^5 M/cm).

2.3.2. Determination of SOD activity

SOD activity was determined by the Misra and Fridovich (1972) method. To 40 μL of supernatant in a 1 cm cuvette was added 260 μL carbonate buffer solution [50 mM sodium carbonate (Sigma-Aldrich) and 0.1 mM EDTA (Vetec)] pH 10.2, plus 200 μL adrenaline (30 mM, Bayer). Absorbance was read at 480 nm after 30 s and 5 min. Enzyme activity was determined using the MEC of SOD (21 M/cm).

2.3.3. Determination of CAT activity

CAT activity was determined by the Radi et al. (1991) method. To 20 mL of supernatant was added 1 mL isolation buffer solution [0.3 M saccharose (Vetec), 1 mL EDTA (Vetec), 5 mM HEPES (Sigma-Aldrich) and 5 mM KH_2PO_4 (Vetec)], plus 0.2 mL of a hydrogen peroxide solution (20 mM, Vetec). Absorbance was read at 240 nm after 0 and 60 s. Results were derived by substituting the absorbance value obtained for each of these times in the formula: CAT concentration = $(A_0–A_{60})$/MEC), where the MEC of H_2O_2 is 0.043 mM/cm. Data were expressed as mM H_2O_2/min/g wet tissue.

2.3.4. Determination of GPx activity

GPx activity was determined by the Gunzler and Flohe-Clairborne (1985) method as modified by Stephensen et al. (2000). To 100 μL of supernatant was added 10 μL glutathione reductase (2 U glutathione reductase, Sigma-Aldrich), plus 290 μL reaction buffer [50 mM K_2HPO_4 (Vetec), 50 mM KH_2PO_4 (Vetec) pH 7.0, 3.5 mM reduced glutathione (Sigma-Aldrich), 1 mM sodium azide (Sigma-Aldrich) and 0.12 mM NADPH (Sigma-Aldrich)] and 100 μL H_2O_2 (0.8 mM, Vetec). Absorbance was read at 340 nm at 0 and 60 s. Enzyme activity was estimated using the equation: GPx concentration = $(A_0 - A_{60})$/MEC), where the MEC of NADPH = 6.2 mM/cm. Results were expressed as mM NADPH/min/g wet tissue.

2.4. Diclofenac and 4 hydroxydiclofenac quantification (LC-MS/MS)

The test systems were the same for sublethal toxicity assays (biomarkers of oxidative stress). Five test systems consisting in $120 \times 80 \times 40$-cm glass tanks filled with water reconstituted added with 7098.4 μg/L equivalent to the lowest observed adverse effect level (LOAEL) and with 6 carp each. These were maintained at room temperature with a natural light/dark photoperiod and provided with constant aeration. A kinetics was run for the following exposure periods: 12, 24, 48, 72 and 96 h. After exposure time, the DCF and 4-OH DCF was determined in each water system and the carp.

The DCF and 4-OH DCF concentration was determined by using an Agilent HPLC (Infity 1290). The Eclipse Plus C18 RRHD (2.1×50 mm, 1.8 μm) chromatographic column was used maintained at 40 °C. The mobile phase that was employed was a 50:50 v/v mixture of acetonitrile and ammonium formate (10 mM). The flow was 0.3 mL min^{-1}, run time 1.8 min and injection volume 2 μL.

The DCF and 4-OH DCF were identified and quantified by means of a mass spectrometer, Triple quadrupole 6430, fitted with electrospray ionization (ESI). The ESI positive mode was used throughout. The optimization of the mass spectrometer was conducted by direct infusion of a 10 ug mL^{-1} standard solution of DCF and 4-OH DCF; thereafter the ionization mode and the precursor ion mode were selected.

2.5. Statistical analysis

In the acute toxicity assay (96-h LC_{50} of DCF), Probit analysis was performed and significance assessed by the degree of 95% LC_{50} overlap (EPA Analysis Program v1.5). The χ^2 linear adjustment test was not significant at $P < 0.05$.

In the sublethal toxicity assays, statistical evaluation of results was done with one-way analysis of variance (ANOVA) and differences between means were compared using the Tukey-Kramer multiple comparisons test, with P set at < 0.05. Statistical determinations were made with the SPSS v10 software package (SPSS, Chicago IL, USA).

3. Results

3.1. Determination of LC_{50}

The 96-h LC_{50} of DCF in *C. carpio* was 70.98 mg/L with a 95% confidence interval of (51.66–98.14). The χ^2 linear adjustment test was not significant at $P < 0.05$.

3.2. Evaluation of oxidative stress

3.2.1. LPX

LPX results are shown in Fig. 1. Carp exposed to 7.098 mg/L of DCF showed a significant increase in this biomarker at 12 h in liver, and 48 and 96 h in gill ($P < 0.05$) by comparison to the

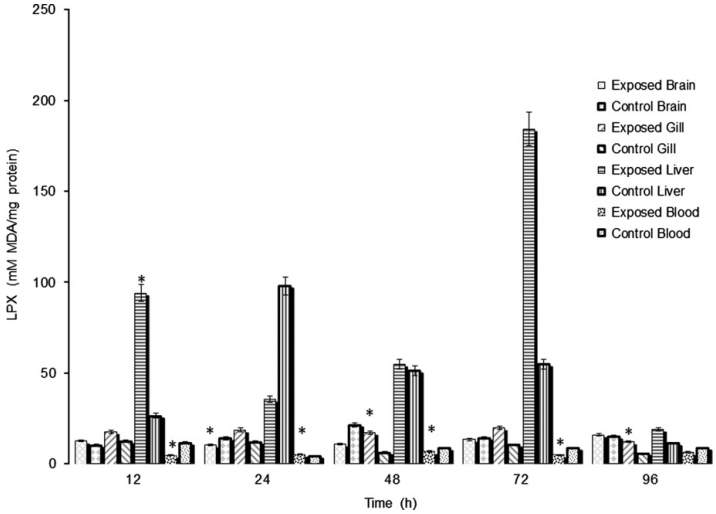

Fig. 1. Lipid peroxidation in brain, gill, liver and blood of *C. carpio* after exposure to DCF for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$).

control group, while a statistically significant reduction was found at 24 h in brain and at 12, 24, 48 and 72 h in blood.

3.2.2. SOD activity

SOD activity results are shown in Fig. 2. Fish exposed to 7.098 mg/L of DCF showed a significant reduction in this activity at 48 h in brain and at 12 h in blood, while a significant increase was found at 72 h in blood, 12 h in liver, and 24, 48 and 96 h in gill ($P < 0.05$), with respect to the control group.

3.2.3. CAT activity

Results of CAT activity are shown in Fig. 3. Carp exposed to 7.098 mg/L of DCF showed a significant increase of this activity at 12 and 48 h in gill and 72 h in liver ($P < 0.05$) with respect to the control group, as well as reduced activity at 12 and 72 h in blood. In reference to carp not exposed to DCF, a significant decrease of brain CAT activity was observed with respect to brain control group ($P > 0.05$) in different exposure times.

3.2.4. GPx activity

GPx activity results are shown in Fig. 4. In fish exposed to 7.098 mg/L of DCF a significant increase occurred at 12, 72 and 96 h in liver ($P < 0.05$) as well as reduced GPx activity at 12 h and 96 h in gill, compared to the control group. GPx activity in brain and blood did not show a significant difference with respect to brain and blood control group ($P > 0.05$) in different exposure times. Only in 12 h a significant increase was observed in brain in the exposed system with respect to control groups ($P < 0.05$).

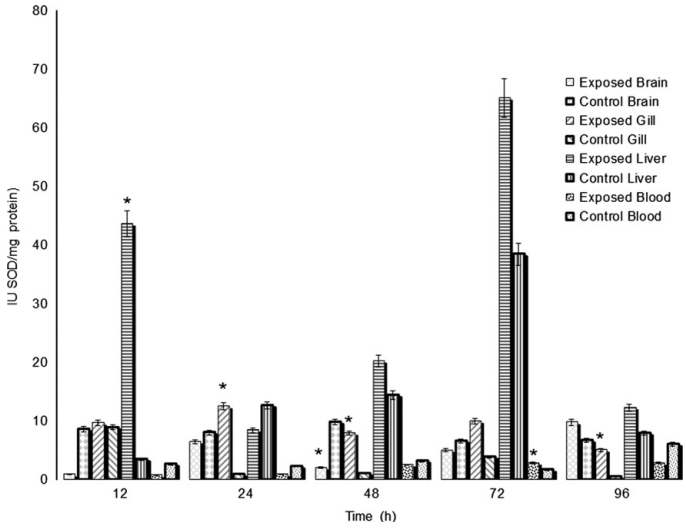

Fig. 2. Superoxide dismutase (SOD) activity in brain, gill, liver and blood of *C. carpio* after exposure to DCF for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. * Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$).

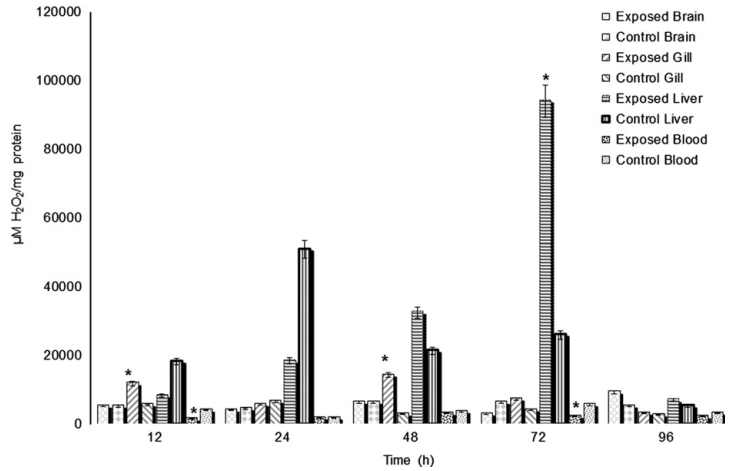

Fig. 3. Catalase (CAT) activity in brain, gill, liver and blood of *C. carpio* after exposure to DCF for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. *Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$).

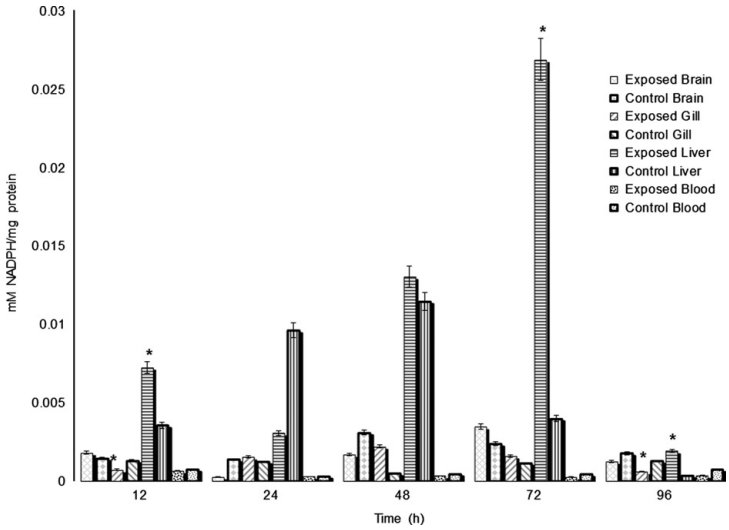

Fig. 4. Glutathione peroxidase (GPX) activity in brain, gill, liver and blood of *C. carpio* after exposure to DCF for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. * Significantly different from control values, ANOVA and Tukey–Kramer ($P < 0.05$).

Table 1
DCF and 4-OH DCF concentrations in the exposure times.

Exposure time (h)	DCF in water system (μg/L)	DCF in carp (μg/L)	4-OH DCF in carp (μg/L)
0	7098.4 ± 354.9	0	0
12	1852.1 ± 55.5	3147.7 ± 75.3	2008.5 ± 100.4
24	1766.6 ± 125.7	3095.1 ± 80.9	2023.7 ± 101.1
48	1610.3 ± 64.4	3215.8 ± 79.7	2075.2 ± 145.2
72	1519.3 ± 60.7	3347.4 ± 65.7	2125.6 ± 127.5
96	1392.6 ± 58.7	3416.4 ± 80.3	2216.3 ± 177.3

Values are the mean of five replicates ± SE.

3.3. *DCF and 4-OH DCF quantification*

Table 1 shows both the DCF and 4-OH DCF concentrations in the water system and carp. As can be seen, the DCF concentrations in the water system are decreasing over time and increasing in the carp. Also the table shows that at 12 h the DCF biotransformation to 4-OH DCF in the carp begins.

4. Discussion

The 96-h LC_{50} of DCF in *C. carpio* was determined to be 70.98 mg/L with a 95% confidence interval of (51.66–98.14). In other species, such as *Lepomis macrochirus* LC_{50} values of ibuprofen have been found 173.0 mg/L (Halling-Sorensen et al., 1998) and in *Oryzias latipes* LC_{50} values of indomethacin of 81.92 mg/L. The LC_{50} value of DCF in *C. carpio* was lower than ibuprofen and indomethacin in other fish species. Therefore DCF could be considered more toxic that other NSAID drugs. These differences may be due to the different biotransformation metabolites of each NSAID compared. e.g. the high toxicity of DCF is due to benzoquinonimine intermediates (Stülten et al., 2008).

EU guidelines (93-67-EEC) laid down by the Commission of the European Communities (1996) classify substances by risk category according to their LC_{50}. Thus, substances with an LC_{50} of < 1 mg/L are extremely toxic, 1–10 mg/L are toxic and 10–100 mg/L hazardous for aquatic organisms. Based on this ranking system, DCF is hazardous for *C. carpio*.

The LC_{50} of DCF in *C. carpio* determined in the present study may be explained by the fact that this pharmaceutical agent acts by blocking the enzyme cyclooxygenase, which catalyzes arachidonic acid degradation during prostaglandin production (Morrow and Roberts, 2001). Prostaglandins are involved in pain mediation in addition to neurotransmission, regulation of systemic circulation, vascular permeability, ion transport across cell membranes, and particularly renal function (Arkhipova et al. 2005). Alterations in any of these processes may have contributed to specimen mortality in our study.

This mechanism of action of DCF has been found in other aquatic species. Hoeger et al. (2005) showed that DCF can inhibit cyclooxygenase activity and therefore also synthesis of prostaglandin E2 in brown trout head kidney macrophagesin vitro, thus confirming the existence in fish of the same mode of action reported previously in mammalian species.

DCF has been identified and quantified in diverse water bodies. It is biodegradable by microorganisms or photodegradable into the following metabolites: 5,4′-dihydroxy-diclofenac, 3- dihydroxy-diclofenac, 4′-dihydroxymethyl-diclofenac, 3′-hydroxymethyl-diclofenac, 4′-hydroxy-diclofenac and 5′-hydroxy-diclofenac (Deng et al., 2003). The latter two are oxidized to intermediates of benzoquinone imine, compounds that are highly toxic to aquatic organisms (Oviedo-Gómez et al., 2010).

In addition to these abiotic transformations, DCF is biotransformed by P450 enzymes present in the smooth endoplasmic reticulum of cells in different organs such as the liver, kidneys, gills, gut, brain, heart and gonads, among others (Stegeman and Livingstone, 1998).

In fish, different families of genes of the P450 complex have been characterized such as CYP1, CYP2, CYP3, CYP4, CYP11, CYP17 and CYP19 (Stegeman and Livingstone, 1998). Also, studies by the Washington University Zebrafish Genome Resources Project report the presence of multiple genes coding for the enzyme

UDP-glucuronosyltransferase (UDPGT) in fish (George and Taylor, 2002).

It is known that in humans the biotransformation of DCF by hydroxylation to form 4-hydroxy-diclofenac is mediated by the CYP2C9 family, while the families CYP2C8, CYP2C18, CYP2C19 and CYP2B6 mediate DCF biotransformation to 5-hydroxy-diclofenac (Tang, 2003). As can be seen, these same P450 families are also present in fish and may be responsible for the biotransformation of DCF to hydroxylated metabolites.

Various studies suggest that in DCF hydroxylation metabolism by the P450 complex, diverse ROS such as the superoxide anion radical (O^{2*}) may be produced in different organ systems. Also, the DCF metabolites 4-OH-diclofenac and 5-OH-diclofenac can be oxidized to intermediates of a reactive quinone imine that reacts with protein nucleophilic groups to form adducts. There is also formation of DCF cationic radicals to nitroxides and quinolone imines, both of which are associated with the redox cycle (Hoeger et al., 2008).

As is evident in Fig. 1, significant differences in LPX compared to the control group ($P<0.05$) occurred generally at all exposure times. However, the organs most affected in evaluating this biomarker were the gills and liver. LPX involves a chain of oxidation reactions, particularly in polyunsaturated fatty acids, which are highly sensitive to ROS-induced oxidation due to the presence of double bonds in their structure. The final step in the LPX process is formation of lipid hydroxyperoxides that can readily break down into various chemical species such as lipid alkoxyl radicals, aldehydes (including MDA), alkanes, lipid epoxides, and alcohols, most of which are toxic and mutagenic products (Porter et al., 1995).

The increased LPX found in liver of *C. carpio* in our study may be explained by the fact that this organ plays an essential role in the biotransformation of xenobiotic compounds (Figueiredo-Fernandes et al., 2006). During biotransformation of DCF by the P450 complex, an oxygenated intermediate—the oxy-cytochrome P450 complex [P450 (Fe^{3+}) 0^{2*}]—is produced which, being highly unstable, may release the superoxide anion (Doi et al., 2002), responsible in turn for LPX.

In freshwater fish, the gills have a major role in the transport of O_2 and other ions such as Na^+, K^+ and Cl^- in order to maintain acid–base homeostasis, osmotic pressure of the body, and regulation of water influx and ion efflux. Due to their intimate contact with water, fish gills are likely to be the main target organ for aquatic pollutants (Monteiro et al., 2005). These organs are also known to be the site of enzyme activity potentially favoring DCF oxidative metabolism and thus promoting production of the ROS responsible for LPX increases in our study.

The phospholipid membrane of aerobic organisms is constantly subjected to oxidation by both exogenous and endogenous forces (Dix and Aikens, 1993).

As shown in (Figs. 1–4) in almost all exposure times no significant differences in biomarkers of brain oxidative stress were found. The brain and nervous system are prone to oxidative stress, and are inadequately equipped with antioxidant defense systems to prevent oxidative damage (Halliwell, 2006). The DCF low octanol/water distribution coefficient (1.22) (Hoeger et al., 2008) makes it difficult to cross hematoencephalic barrier and hence oxidative stress was not found in this organ.

Hoeger et al. (2008) demonstrated that DCF is not completely excreted through first pass metabolism in brown trout, but that a significant part of the applied DCF enters enterohepatic circulation. The resulting prolonged availability of DCF in the organism possible promotes accumulation of DCF, despite its basically low tendency for bioaccumulation as judged by its octanol/water partition coefficient. In our study the blood LPX results showed a tendency to significant decrease with respect to control ($P<0.05$). The prolonged accumulation in different tissues (as gill and liver) of DCF and its metabolites and blood disappearance could be responsible for the decrease of both blood LPX and CAT activity.

Antioxidant defenses may be induced by diverse environmental contaminants (Vlahogianni et al., 2007). SOD is the first mechanism of antioxidant defense and the main enzyme responsible for offsetting the toxic effects induced by the presence of ROS. This is particularly so in the case of the superoxide ion, which is a minor product of mitochondrial respiration (van der Oost et al., 2003) and is biotransformed by SOD to hydrogen peroxide, after which, CAT and GPx take part in the capture and later dismutation of H_2O_2 to H_2O (Barata et al., 2005). In our study, SOD activity increased with respect to controls ($P<0.05$) at different exposure times in liver and gill, while in brain it showed a tendency to decrease at different exposure times up to 72 h. The significant increase in this activity in liver and gill may be explained by the fact that DCF oxidative metabolism favors formation of the O^{2*} anion, which is responsible for the increase in this biomarker.

Furthermore, the nervous system is particularly vulnerable to ROS due to its high consumption of O_2 since neuronal membranes are rich in polyunsaturated fatty acids, in addition to the fact that iron is present in significant amounts and may catalize free radical reactions (Halliwell, 2006). While DCF has a log Kow value of 0.7 and therefore low lipophilicity, it is a small molecule that can readily pass through membranes (Hoeger et al., 2005). Reduced SOD activity in brain of *C. carpio* may result from the fact that several DCF metabolites bind to proteins and inhibit their activity. Likewise, NSAIDs—including DCF among others—affect the mitochondrion and consequently oxidative phosphorylation, thus potentially increasing ROS production. This is particularly true of the O^{2*} anion (Asensio et al., 2007) which, added to the ROS produced by natural processes in the brain, can potentially block SOD activity.

Both CAT and GPx activities were also significantly increased in gill and liver. Bagnyukova et al. (2006) state that LPX products appear to be involved in the regulation of several antioxidant enzymes. Thus, LPX increases in the present study may also explain the observed increased activity of antioxidant enzymes.

In conclusion, DCF induces oxidative stress on *C. carpio*, particularly in organs such as the liver and gill. The set of biomarkers used in the present study is therefore a reliable early marker for toxicity assessment in aquatic species.

Acknowledgments

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Effect of ibuprofen in blood, gill, liver and brain on common carp (Cyprinus carpio) using oxidative stress biomarkers

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Abstract:	Although trace concentrations of ibuprofen (IBP) have been detected in diverse water bodies, there is currently insufficient information on the potentially deleterious effects of this xenobiotic. The present study aimed to determine whether IBP induces oxidative stress in brain, liver, gill and blood of the common carp Cyprinus carpio. To this end, the median lethal concentration at 96-h (96-h LC50) was determined and the lowest observed adverse effect level (LOAEL) was established. Carp were exposed to the latter concentration (17.55 mg L-1) for 12, 24, 48, 72 and 96 h, and the following biomarkers were evaluated: lipid peroxidation (LPX) and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Results indicate that LPX and antioxidant enzymes activity increased significantly ($p<0.05$) with respect to the control group in liver, gill and blood, while no significant differences occurred in brain. In conclusion, IBP induces oxidative stress on C. carpio, the liver being the organ most affected by this damage.
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Effect of ibuprofen in blood, gill, liver and brain on common carp (*Cyprinus carpio*) using oxidative stress biomarkers

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Abstract

Although trace concentrations of ibuprofen (IBP) have been detected in diverse water bodies, there is currently insufficient information on the potentially deleterious effects of this xenobiotic. The present study aimed to determine whether IBP induces oxidative stress in brain, liver, gill and blood of the common carp *Cyprinus carpio*. To this end, the median lethal concentration at 96-h (96-h LC_{50}) was determined and the lowest observed adverse effect level (LOAEL) was established. Carp were exposed to the latter concentration (17.55 mg L^{-1}) for 12, 24, 48, 72 and 96 h, and the following biomarkers were evaluated: lipid peroxidation (LPX) and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Results indicate that LPX and antioxidant enzymes activity increased significantly (p<0.05) with respect to the control group in liver, gill and blood, while no significant differences occurred in brain. In conclusion, IBP induces oxidative stress on *C*. *carpio*, the liver being the organ most affected by this damage.

Keywords

Cyprinus carpio; ibuprofen; lipid peroxidation; superoxide dismutase; catalase; glutathione peroxidase

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are considered one of the most frequently used groups of pharmaceutical agents at world level. Takagi et al. (2006) state that these pharmaceuticals account for more than 70 million annual prescriptions in Great Britain, Spain and Japan. Furthermore, they rank sixth in worldwide sales volume among pharmaceutical groups, with an annual production of several thousand tons (Parolini et al. 2009).

NSAIDs are pharmaceutical agents with diverse anti-inflammatory, analgesic and antipyretic properties. Chemically, they are a heterogeneous group and are not closely related in terms of structure; however, they have in common various therapeutic actions and adverse effects (Hardman et al. 2003).

The most commonly used pharmaceuticals within this group are acetylsalicylic acid (ASA), paracetamol (PAR), diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX) (Katzung 2007). IBP has an effective anti-inflammatory, antipyretic and analgesic action. It is used to relieve muscle pain and various other inflammatory disorders (Mendez-Arriaga et al. 2008; Scheytt et al. 2005). In Mexico IBP is sold over the counter and was in 2008 one of the six most frequently sold pharmaceutical agents, its use exceeding 2000 kg per year (0.02 per capita per year). IBP acts by inhibiting the cyclooxygenase enzyme system, particularly COX-1 (constitutive) and COX-2 (induced at the site of inflammation). These enzymes are responsible for converting arachidonic acid to prostaglandins and thromboxanes, which are mediators involved in different homeostatic processes throughout the body (Parolini et al. 2009; Richards and Cole 2006; Hardman et al. 2003; Morrow and Roberts 2001).

IBP has been detected in water bodies at diverse concentrations. It has been found in surface water at concentrations of 0.003-0.25 μg L^{-1} (Cordy et al. 2004) and 3.08 μg L^{-1} (Bound and Voulvoulis 2005), in sewage sludge and biosolids at 0.12 μg L^{-1} (Ternes et al. 2004), in ground water at 0.129 μg L^{-1} (Verstraeten et al.2005) and 0.012 μg L^{-1} (Kreuzinger et al. 2004), and in drinking water at 0.009 μg L^{-1} (Vieno et al. 2005). IBP has also been identified and quantified in wastewater treatment plant affluents and effluents at concentrations of 1.1-151 μg L^{-1} (Gómez et al. 2006), 7.741-33.764 μg L^{-1} (Roberts and Thomas 2006), 2.235-6.718 μg L^{-1} (Verenitch et al. 2006), 4.1-10. μg L^{-1} (Lee et al. 2005) and 1.43 μg L^{-1} (Santos et al. 2005).

In Mexico information on the concentrations of this group of contaminants in different aquatic systems is scarce. A study by Siemens et al. (2008) determined the presence and diverse concentrations

of pharmaceutical compounds in treated and untreated wastewater as well as sewage sludge from the Mexico City metropolitan area. Prominent among the pharmaceutical agents identified were NSAIDs, and within this group, IBP at concentrations of 3.5 µg L^{-1}.

Diverse studies have described the toxic effects of trace concentrations of IBP. Méndez-Arriaga et al. (2008) described IBP-induced toxicity in rainbow trout (*Oncorhynchus mykiss*). Richards and Cole (2006) found thoracic malformations associated with exposure to IBP at concentrations of 1 to 100 mg L^{-1} in *Xenopus laevis*. IBP also significantly affects growth in several species of bacteria and fungi (Pomati et al. 2006; Sanyal et al. 1993).

One way to evaluate the damage induced by emerging contaminants present in water bodies is the use of biomarkers, which are defined as any measurable biochemical, physiological or morphologic changes associated with exposure to a toxic agent.

Oxidative stress, considered one of the major mechanisms of action of toxicants, is among the most frequently used biomarkers since it is able to evaluate damage in general to biomolecules such as lipids, proteins and DNA (Barata et al. 2005). Regulated production of free radicals and maintenance of redox homeostasis are essential for the physiological health of organisms. During metabolic processes, a small proportion (2-3%) of free radicals and reactive oxygen species (ROS) is able to elude the action of antioxidant mechanisms, inducing oxidative damage on cell components. The imbalance between production and neutralization of ROS by antioxidant mechanisms within the body is called oxidative stress (Davies 1995).

The toxic effects of many xenobiotics are mediated by ROS formation as a result of redox cycling (Abdollahi et al. 2004).The failure of antioxidant defenses to detoxify excess ROS production may lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (LPX) (Halliwell and Gutteridge 1999). LPX in particular is considered the primary mechanism through which oxyradicals potentially damage tissues, hampering cell function and altering the physicochemical properties of the cell membrane, leading to disruption of vital functions (Rikans and Hornbrook 1997).

The balance between endogenous or exogenous prooxidant factors (environmental pollutants) and enzymatic and non-enzymatic antioxidant defenses in organ systems can be used to assess toxicity under stressful environmental conditions, particularly oxidative damage induced by different kinds of chemical pollutants. Oxidative damage to lipids, proteins and DNA, and adverse effects on enzymatic

antioxidant defense mechanisms in aerobic organisms have been used in recent years as biomarkers for monitoring environmental pollution (Valavanidis et al. 2006). The most important oxidative stress biomarkers used in toxicological studies of aquatic systems are LPX, hydroperoxide content, protein oxidation, and enzymatic antioxidant defenses (Dröge 2003).

Diverse bioindicators can be used to evaluate the toxic impact of contaminants present in water bodies. Aquatic indicator organisms are selected taking into account their filtration capacity, ease of culture, and sensitivity to diverse contaminants (Livingstone et al. 1994). Toxicity studies in teleost fishes are necessary in order to understand the mechanisms of action of contaminants and to predict and evaluate their toxicity, particularly when contamination is chronic.

The common carp *Cyprinus carpio* is commonly used as a bioindicator species (Huang et al. 2007) since cyprinids are quantitatively the most important group of teleost fishes cultured throughout the world for commercial purposes and are also very resistant, easy to maintain organisms. Toxicity assays can be performed under simulated conditions in which the contaminant is added to (water or artificial sediment) systems in order to determine the specific effect of the test substance, or else by using systems containing water or sediment from natural water bodies. The latter procedure permits determination of the toxicity induced by a mixture of contaminants present in the water body as well as of potential interactions between xenobiotics and system components.

The aim of this study was to evaluate the toxicity induced by a sublethal concentration of IBP on various organs and tissues of the freshwater teleost fish *C. carpio*, using oxidative stress biomarkers to assess the potential risk posed by water-borne pharmaceuticals to the physiology and survival of aquatic organisms.

2. Materials and methods

2.1 Test substance

IBP (CAS Number 15687-27-1, >98% purity) was purchased from Sigma-Aldrich Corp. Mexico. IBP (1 g) was dissolved in deionized water to prepare stock solutions.

2.2 Specimen procurement and maintenance

Common carp (*Cyprinus carpio*) 18.39 ± 0.31 cm in length and weighing 50.71 ± 7.8 g were obtained from the aquaculture facility in Tiacaque, State of Mexico. Fish were transported to the laboratory in well-sealed polyethylene bags containing oxygenated water and were subsequently stocked in a large tank with dechlorinated tap water (previously reconstituted with salts) and acclimated to test conditions for 30 days prior to the experiment. During acclimation, carp were fed Pedregal Silver™ fish food, and three-fourths of the tank water was replaced every 24 h in order to maintain a healthy environment. The physicochemical characteristics of tap water reconstituted with salts were maintained, i.e. temperature 20 ± 2 °C, oxygen concentration 80-90%, pH 7.5-8.0, total alkalinity 17.8 ± 7.3 mg L^{-1}, total hardness 18.7 ± 0.6 mg L^{-1}. A natural light/dark photoperiod was maintained.

2.3 Median lethal concentration (LC_{50}) and oxidative stress determination

Test systems consisting in 120 x 80 x 40-cm glass tanks filled with water reconstituted from the following salts: $NaHCO_3$ (174 mg L^{-1}, Sigma-Aldrich), $MgSO_4$ (120 mg L^{-1}, Sigma-Aldrich), KCl (8 mg L^{-1}, Vetec) and $CaSO_4.2H_2O$ (120 mg L^{-1}, Sigma-Aldrich) were maintained at room temperature with constant aeration and a natural light/dark photoperiod. Static systems were used and no food was provided to specimens during the exposure period.

To establish the target concentration to be used for oxidative stress evaluation, the median lethal concentration (LC_{50}) of IBP was determined. To this end, seven experimental systems to which were added different nominal concentrations of IBP (9.5, 18.9, 37.7, 75.2, 150.0, 300.8 and 600 mg L^{-1}) plus an eighth IBP-free control system were set up, and ten carp randomly selected from the stock (using the random number method) were placed in each system. A total of 240 fish were used for LC_{50} determination.

Duration of the exposure period was 96 h, at the end of which the number of dead specimens in each system was counted. The assay was performed in triplicate. The 96-h LC_{50} of IBP and its 95% confidence limits (p<0.05) were estimated by Probit analysis (EPA, v1.5). The data obtained were used to estimate the concentration to be used in the assays for oxidative stress determination.

Sublethal toxicity assays involved adding IBP at a concentration equal to the lowest observed adverse effect level (LOAEL), i.e. 17.557 mg L^{-1}, to five experimental systems with six carp each. A kinetics was run for the following exposure periods: 12, 24, 48, 72 and 96 h.

An IBP-free control system with six carp was set up for each exposure period, and sublethal assays were performed in triplicate. A total of 300 fish were used in the sublethal assays. At the end of the exposure period, fish were removed from the systems and placed in a tank containing 50 mg L^{-1} of clove oil as an anaesthetic (Yamanaka et al. 2011). Anesthetized specimens were placed in a lateral position and blood was removed with a heparinized 1-mL hypodermic syringe by puncture of the caudal vessel performed laterally near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line.

After puncture, specimens were placed in an ice bath and sacrificed. The gill, liver and brain were removed, placed in phosphate buffer solution pH 7.4 and homogenized. The supernatant was centrifuged at 12,500 rpm and –4 ºC for 15 min. The following biomarkers were then evaluated: LPX and activity of the antioxidant enzymes SOD, CAT and GPx. All bioassays were performed on the supernatant.

2.3.1 Determination of LPX

LPX was determined by the Büege and Aust (1978) method. To 100 mL of supernatant was added Tris-HCl buffer solution pH 7.4 (Sigma-Aldrich) until a 1-mL volume was attained. Samples were incubated at 37 °C for 30 min; 2 mL TBA-TCA reagent [0.375% thiobarbituric acid (TBA, Sigma-Aldrich) in 15% trichloroacetic acid (TCA, Sigma-Aldrich)] was added and samples were shaken in a vortex. They were then heated to boiling for 45 min, allowed to cool, and the precipitate removed by centrifugation at 3,000 rpm for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56×10^5 M cm^{-1}).

2.3.2 Determination of SOD activity

SOD activity was determined by the Misra and Fridovich (1972) method. To 40 µL of supernatant in a 1-cm cuvette was added 260 µL carbonate buffer solution [50 mM sodium carbonate (Sigma-Aldrich) and 0.1 mM EDTA (Vetec)] pH 10.2, plus 200 µL adrenaline (30 mM, Bayer). Absorbance was read at 480 nm after 30 s and 5 min. Enzyme activity was determined using the MEC of SOD (21 M cm^{-1}), and results were expressed as IU SOD mg^{-1} protein.

2.3.3 Determination of CAT activity

CAT activity was determined by the Radi et al. (1991) method. To 20 mL of supernatant was added 1 mL isolation buffer solution [0.3 M saccharose (Vetec), 1 mL EDTA (Vetec), 5 mM HEPES (Sigma-Aldrich) and 5 mM KH_2PO_4 (Vetec)], plus 0.2 mL of a hydrogen peroxide solution (20 mM, Vetec). Absorbance was read at 240 nm after 0 and 60 s. The absorbance value obtained for each of these times was substituted in the formula: CAT activity = $(A_0 - A_{60})$/MEC), where the MEC of H_2O_2 is 0.043 mM cm^{-1}, and results were expressed as mM H_2O_2 min^{-1} g^{-1} wet tissue.

2.3.4 Determination of GPx activity

GPx activity was determined by the Gunzler and Flohe-Clairborne (1985) method as modified by Stephensen et al. (2000). To 100 µL of supernatant was added 10 µL glutathione reductase (2 U glutathione reductase, Sigma-Aldrich), plus 290 µL reaction buffer [50 mM K_2HPO_4 (Vetec), 50 mM KH_2PO_4 (Vetec) pH 7.0, 3.5 mM reduced glutathione (Sigma-Aldrich), 1 mM sodium azide (Sigma-Aldrich) and 0.12 mM NADPH (Sigma-Aldrich)] and 100 µL H_2O_2 (0.8 mM, Vetec). Absorbance was read at 340 nm at 0 and 60 s. Enzyme activity was estimated using the equation: GPx activity = $(A_0 - A_{60})$/MEC), where the MEC of NADPH = 6.2 mM cm^{-1}. Results were expressed as mM NADPH min^{-1} g^{-1} wet tissue.

2.4 Statistical analysis

In the acute toxicity assay (96-h LC_{50} of IBP), Probit analysis was performed and significance assessed by the degree of 95% LC_{50} overlap (EPA Analysis Program v1.5). The χ^2 linear adjustment test was not significant at $p<0.05$.

In the sublethal toxicity assays, results were statistically evaluated by one-way analysis of variance (ANOVA) and differences between means were compared using the Tukey-Kramer multiple comparisons test, with p set at <0.05. Statistical determinations were made with the SPSS v10 software package (SPSS, Chicago IL, USA).

3. Results

3.1 Determination of LC_{50}

The 96-h LC_{50} of IBP in *C. carpio* was 175.6 mg $L^{-1}$with a 95% confidence interval of (107.31-334.05). The χ^2 linear adjustment test was not significant at $p<0.05$.

3.2 Evaluation of oxidative stress

3.2.1 LPX

LPX induced by exposure to the LOAEL of IBP is shown in Figure 1. A significant increase ($p\leq0.05$) with respect to the control group was observed in blood at 24 h and gill at 48 and 96 h. The highest increases occurred in liver at 12, 48 and 72 h. No significant differences in this biomarker were found in brain.

3.2.2 SOD activity

Figure 2 shows SOD activity results. Significant increases with respect to the control group ($p<0.05$) occurred in liver at 12 and 24 h, in blood at 24 h, and in gill at 24, 48 and 96 h, while brain showed no significant differences ($p<0.05$) with respect to the control group.

3.2.3 CAT activity

CAT activity, expressed as mM H_2O_2 mg^{-1} protein, is shown in Figure 3. A significant increase ($p<0.05$) with respect to the control group was found in blood at 24 h, in liver at 12, 24 and 96 h, and in gill at 96 h. No significant differences ($p<0.05$) with respect to the control group occurred in brain.

3.2.4 GPx activity

Figure 4 shows GPx activity results. A significant increase with respect to the control group ($p<0.05$) occurred in both blood and gill at 12 h. In liver, there was a significant increase at 12 h and significant reductions at 24 and 96 h. Brain showed no significant differences with respect to the control group.

4. Discussion

The 96-h LC_{50} of IBP obtained in the present study was 175.565 mg L^{-1}, with a 95% confidence interval of (107.31-334.05). This value is similar to that reported by Halling-Sorensen et al. (1998), who found a 96-h LC_{50} of IBP of 173 mg L^{-1}in bluegill sunfish (*Lepomis macrochirus*). IBP toxicity

varies from one species to another and even between strains of the same species: the LC_{50} of IBP was found to be 132.6 mg L^{-1} in *Daphnia magna* at 48 h (Han et al. 2006); 17.1 mg L^{-1} in the mollusc *Planorbis carinatus* (Pounds et al. 2008) and 22.36 mg L^{-1} in *Hydra attenuata (*Quinn et al. 2008) at 72 h; and 142 mg L^{-1} in the fish *Cirrhinus mrigala* at 24 h (Saravanan et al. 2011). These studies indicate that IBP toxicity is variable and that the most resistant species to this xenobiotic is *C*. *carpio*.

The LC_{50} value found in the present study may be due to the fact that NSAIDs, particularly IBP, act through blocking of the enzyme cyclooxygenase (COX) which catalyzes the degradation of arachidonic acid in prostaglandin production (Morrow and Roberts 2001). Prostaglandins are involved in pain management, neurotransmission, blood flow regulation, vascular permeability, ion transport across cell membranes and renal function (Arkhipova et al. 2005). Fish mortality during acute exposure may be due to diverse COX-mediated physiological changes including cardiac anomalies, spinal curvature and behavioral changes (Van Hecken et al. 2000; David and Pancharatna 2009).

Due to the presence of sunlight, NSAIDs such as DCF and PAR are photodegradable in water bodies, resulting in formation of smaller, more hydrophobic molecules reported to be more toxic to a variety of organisms (Islas-Flores et al. 2013; Gómez-Oliván et al. 2012; Oviedo-Gómez et al. 2010). Diverse studies state that IBP is resistant to direct photodegradation because its absorption spectrum does not coincide with the solar radiation spectrum. However, the presence of humic acids from organic matter increases the degree of photodegradation in water bodies.

The main metabolites of IBP photodegradation in water bodies are 4-isobutylacetophenone, 1-(6-methoxy-2-naphthyl)ethanol and 2-acetyl-6-methoxy naphthalene (Miranda et al. 1991). IBP is also biodegradable in water bodies due to the presence of microorganisms. The main metabolites formed by bacterial degradation of IBP are 1-hydroxy ibuprofen, 2-hydroxy ibuprofen, 1,2-dihydroxy ibuprofen (Marco-Urrea et al. 2009), S-ibuprofenol and ibuprofenol acetate (Chen and Rosazza 1994).

In addition to abiotic transformations in the experimental system, IBP is biotransformable by the cytochrome P450 (CYP) present in the body. In humans, Phase 1 metabolism of R(-)- and S(+)-ibuprofen involves hydroxylation of the isobutyl chain to 2- or 3-hydroxy- derivatives and subsequent oxidation to 3-carboxy-ibuprofen and p-carboxy-2-propionate, these oxidative reactions being catalyzed by CYP2C8 and CYP2C9 (Graham and Williams 2004). After administration, IBP is eliminated in a mixture of parent compound and metabolites. In humans, only 15% of the ingested IBP is eliminated as parent compound, while 26% is eliminated as hydroxy- and 43% as carboxy-ibuprofen,

including conjugates. Furthermore, other authors report virtually no elimination of unchanged ibuprofen, and 26% and 35% elimination of hydroxy- and carboxy- metabolites and their conjugates, respectively (Mills et al. 1973).

In fish, CYP enzymes are found in the smooth endoplasmic reticulum of cells of different organs such as the liver, kidney, gill, gut, brain, heart and gonads (Stegeman and Livingstone 1998), but the liver is where CYP-mediated Phase-1 reactions of the majority of endogenous and exogenous subtrates occur (Cok et al. 1998). Different CYP gene families have been characterized in diverse fish species, including CYP1, CYP2, CYP3, CYP4, CYP11, CYP17 and CYP19 (Stegeman and Livingstone 1998). Various studies suggest that fish are able to metabolize IBP in a manner similar to mammals (Davies 1998). Gomez et al. (2011) observed that 2-hydroxy ibuprofen was the major metabolite identified in *in vitro* metabolism studies on fish. However, levels of this metabolite were still below parent IBP levels. Other hydroxylated metabolites were not identified in the latter study and the CYP1A subfamily appeared to be involved in IBP metabolism in fish.

In fish, as in other aerobic organisms, an antioxidant defense system matches the toxic action of a prooxidant system. However, when this balance is disturbed in the prooxidant direction, oxidative stress occurs inducing oxidative damage on cells (Halliwell and Gutteridge 1999). The most studied, harmful consequence of ROS action on living organisms is LPX: a series of free radical chain reactions leading to breakdown of polyunsaturated fatty acids, which destroys cell membranes and causes cell death (Ognjanovic et al. 2008).

In the present study, a significant increase in LPX with respect to the control group ($p<0.05$) occurred in blood, liver and gill, this damage being most evident in liver at 12 h (Fig. 1). These results may be due to the fact that the toxicity of pharmaceuticals is mediated by ROS formation as a result of biotransformation of these compounds through redox cycling (Ahmad et al. 2000; Abdollahi et al. 2004). The most frequently used organ in oxidative stress studies is fish liver, which is the most metabolically active tissue and the site where most biotransformation-related changes occur (van der Oost et al. 2003). CYP is known to produce an oxygenated intermediate — the oxy-cytochrome P450 complex [P450 (Fe^{3+}) 0_2^-] – during the biotransformation of NSAIDs, with subsequent release of the superoxide anion by reaction decoupling (Doi et al. 2002), and this may explain the increase in LPX observed in this organ in the present study.

In freshwater fish, the gills have a major role in the transport of O_2 and other ions such as Na^+, K^+ and Cl^- in order to maintain acid-base homeostasis, osmotic pressure of the body, and regulation of water influx and ion efflux. Due to their intimate contact with water, fish gills are probably the main target organ for aquatic pollutants (Monteiro et al. 2005). These organs are also known to be the site of enzyme activity potentially favoring oxidative metabolism and thus promoting production of the ROS responsible for LPX increases.

Brain was the only organ in which no significant differences with respect to the control group (p<0.05) were observed at any exposure time (Figs. 1-4). The nervous system is particularly vulnerable to ROS as a result of its use of large quantities of O_2 and because neuronal membranes are rich in polyunsaturated fatty acids. This organ is also rich in iron which can catalyze free radical reactions (Carey 2002; Halliwell 2006; Spencer et al. 1998). However, due to its partition coefficient (log Kow = 3.97) and low lipophilicity (US EPA 2009), IBP cannot easily cross the hematoencephalic barrier and is unable to penetrate into the brain and induce oxidative damage.

Diverse environmental contaminants can induce antioxidant defenses (Vlahogianni et al. 2007). SOD is the first mechanism of antioxidant defense and the main enzyme responsible for offsetting the effects of ROS, particularly the superoxide ion (van der Oost et al. 2003) which is converted to hydrogen peroxide by SOD. Subsequently, H_2O_2 is sequestered and degraded to H_2O by CAT and GPx. In the present study, SOD activity increased in liver, blood and gill with respect to the control group (p<0.05), the organ recording the highest increase being liver at 12 h. This increase may be due to the fact that during the CYP-mediated biotransformation of IBP in carp, the superoxide anion radical O^{2*}, responsible for increased SOD activity, is released (Doi et al. 2002).

Since NSAIDs – including IBP – affect the mitochondrion and consequently also oxidative phosphorylation, increased ROS production, particularly of $O^{\bullet}_{2-}$, may occur resulting in increased SOD activity and higher levels of hydrogen peroxide (Asensio et al. 2007), as evident in the present study with exposure of *C. carpio* to this compound.

Significant increases with respect to the control group (p<0.05) also occurred in both CAT and GPx activity (Figs. 3 and 4), the highest increase in both cases being observed in liver at 12 h. This may be explained by the fact that some xenobiotics, including NSAIDs, are biotransformable by CYP (Parolini et al. 2009).

As stated above, in the present study an increase in SOD activity occurred in *C. carpio* exposed to IBP, leading to increased hydrogen peroxide formation, which may act as a signal for CAT and GPx bioactivation in order to convert this highly toxic free radical to less toxic compounds. Similar responses have been found in other aquatic organisms exposed to other NSAIDs (Oviedo-Gómez et al. 2010; Gómez-Oliván et al. 2012; Islas-Flores et al. 2013).

In conclusion, IBP induces oxidative stress on liver, blood and gill of *C. carpio*, this damage being most evident in liver. In brain, no significant differences were observed with the biomarkers used. Oxidative stress is a reliable biomarker for evidencing exposure to sublethal concentrations of NSAIDs such as IBP.

Acknowledgments

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Figure captions

Fig. 1 Lipid peroxidation (LPX) in brain, gill, liver and blood of *C*. *carpio* exposed to ibuprofen for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. PT = protein. *Significantly different from control values, ANOVA and Tukey-Kramer (p<0.05)

Fig. 2 Superoxide dismutase (SOD) activity in brain, gill, liver and blood of *C*. *carpio* exposed to ibuprofen for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. PT = protein. *Significantly different from control values, ANOVA and Tukey-Kramer (p<0.05)

Fig. 3 Catalase (CAT) activity in brain, gill, liver and blood of *C*. *carpio* exposed to ibuprofen for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. PT = protein. *Significantly different from control values, ANOVA and Tukey-Kramer (p<0.05)

Fig. 4 Glutathione peroxidase (GPX) activity in brain, gill, liver and blood of *C*. *carpio* exposed to ibuprofen for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. PT = protein. *Significantly different from control values, ANOVA and Tukey-Kramer (p<0.05)

Figure 1
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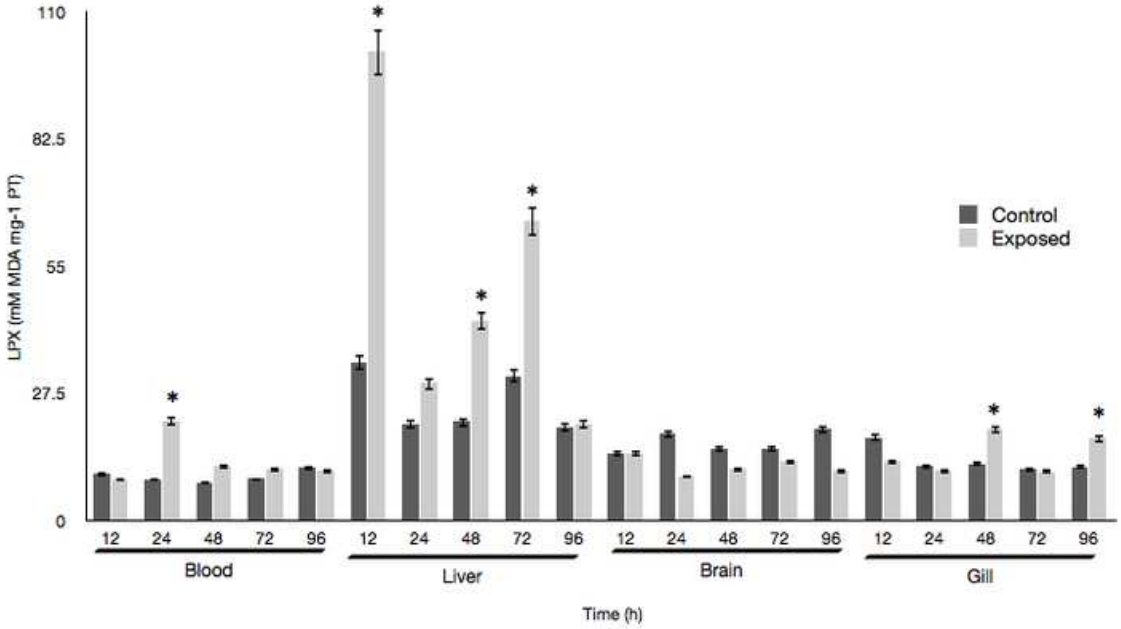

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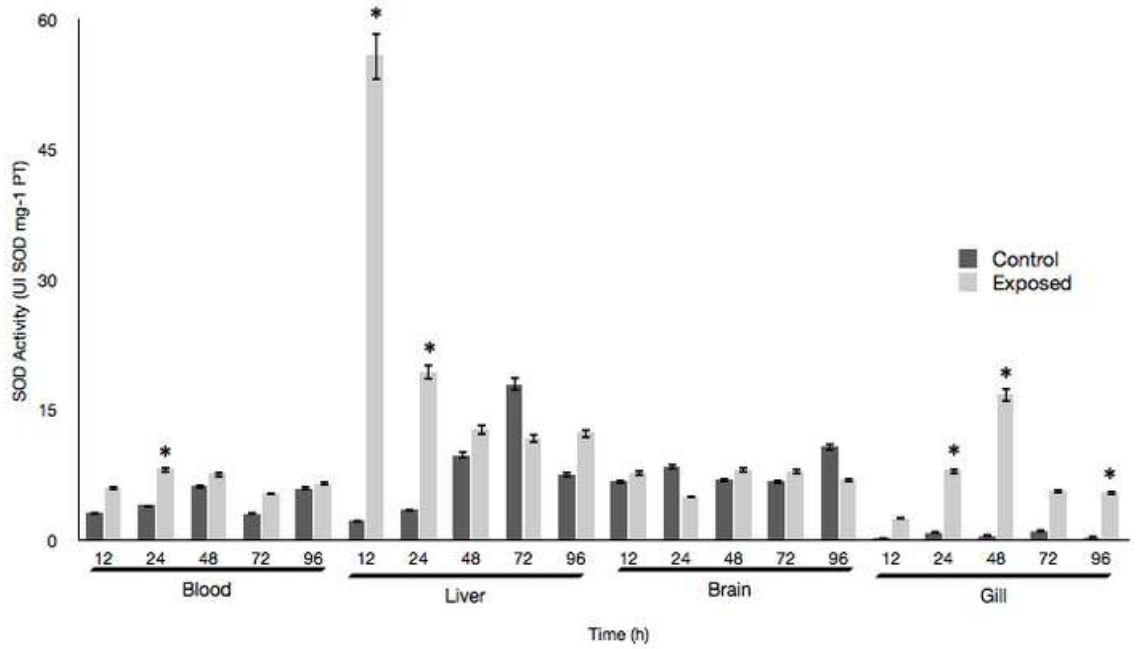

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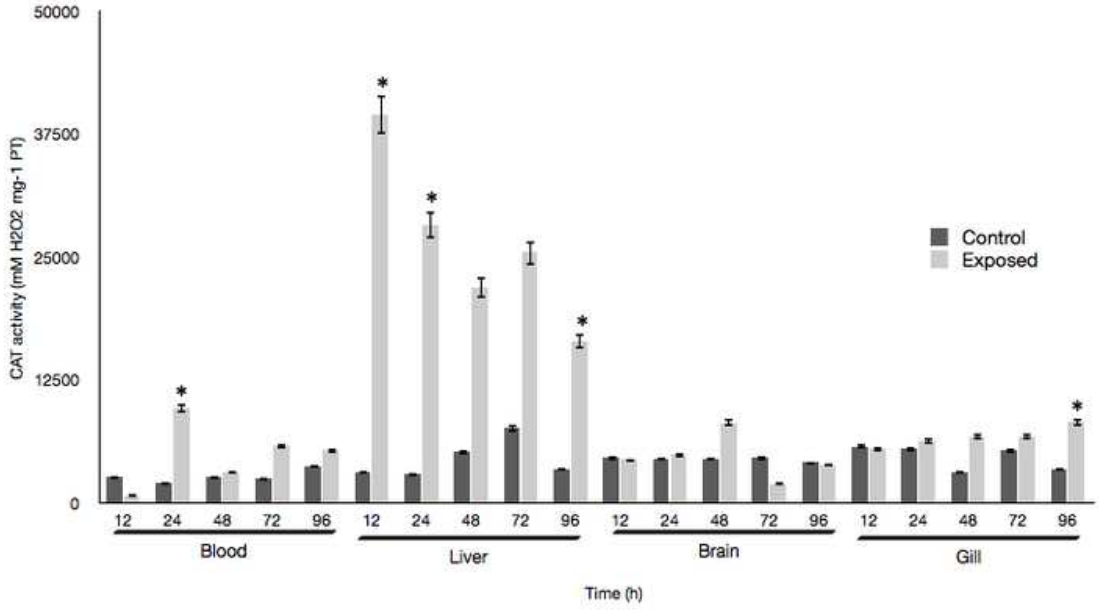

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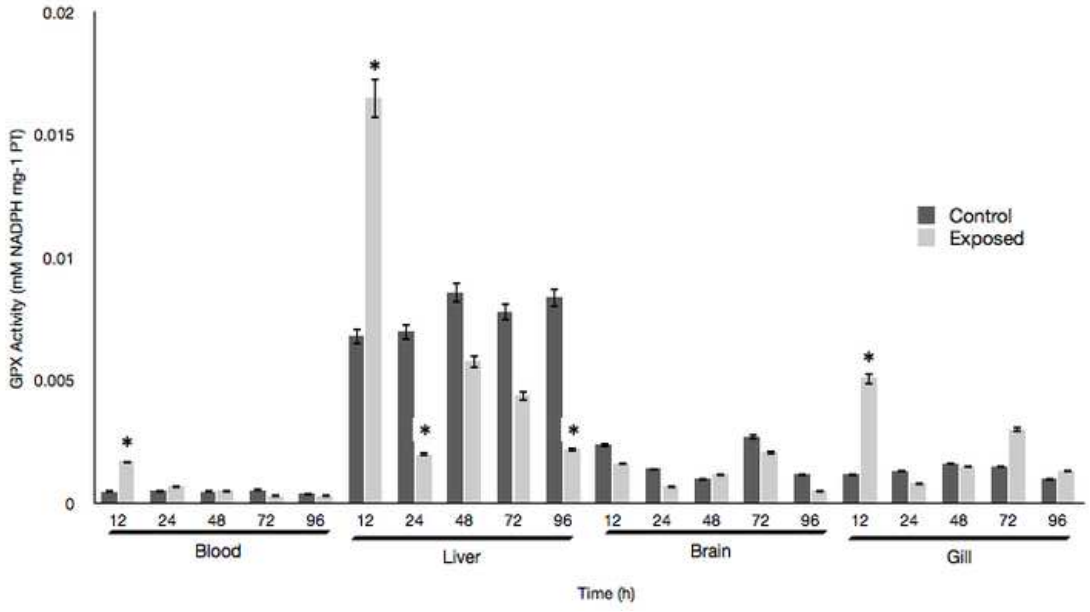

8. Anexos

8.1 Resultados de la mezcla IBP-DCF

8.1.1 Determinación del grado de lipoperoxidación

La peroxidación lipídica causa deterioro en el funcionamiento de la membrana biológica, disminuye la fluidez, inactiva las enzimas unidas a la membrana y los receptores, y puede cambiar inespecíficamente la permeabilidad a los iones de calcio (Bast, 1993). El MDA , producto final de la peroxidación lipídica, es un buen marcador de daño mediado por radicales libres y estrés oxidativo (Del Rio et al., 2005). La cantidad de MDA generado en el grupo control y en los organismos expuestos a la mezcla IBP-DCF se muestra en la figura 1.

De forma general se puede observar que en la mezcla aumenta significativamente el grado de lipoperoxidación en cerebro a las 12, 24, 72 y 96 h con respecto al grupo control ($p < 0.05$), en sangre se muestra este aumento significativo respecto al control ($p < 0.05$) a las 12, 48 y 72 h, en hígado este incremento se presenta a las 24 h, mientras que a las 48 y 72 h en este mismo órgano se reduce significativamente el daño con respecto al grupo control ($p < 0.05$). Como se muestra en la figura, las branquias presentan el mayor daño por lipoperoxidación, se puede observar que a las 24, 48, 72 y 96 h se presenta un incremento significativo con respecto al grupo control ($p < 0.05$). Este incremento puede ser explicado por el metabolismo oxidativo de los NSAID. Se sabe que mediante la biotransformación de fase I de los NSAID el citocromo P450 (CYP) produce un intermediario oxigenado, que es el complejo de oxicitocromo P450 [P450 (Fe^{3+}) 0_2^-], que durante la biotransformación de estos fármacos sufre un desacoplamiento con la consecuente liberación del radical anión superóxido (Doi et al., 2002), que es una sustancia oxidante capaz de lipoperoxidar a los lípidos de membrana. Además durante la biotransformación del DCFse forman 4'hidroxidiclofenaco y 5'hidroxidiclofenaco, que generan como intermediario a la benzoquinoimina, la cual puede incrementar la formación de EROs.

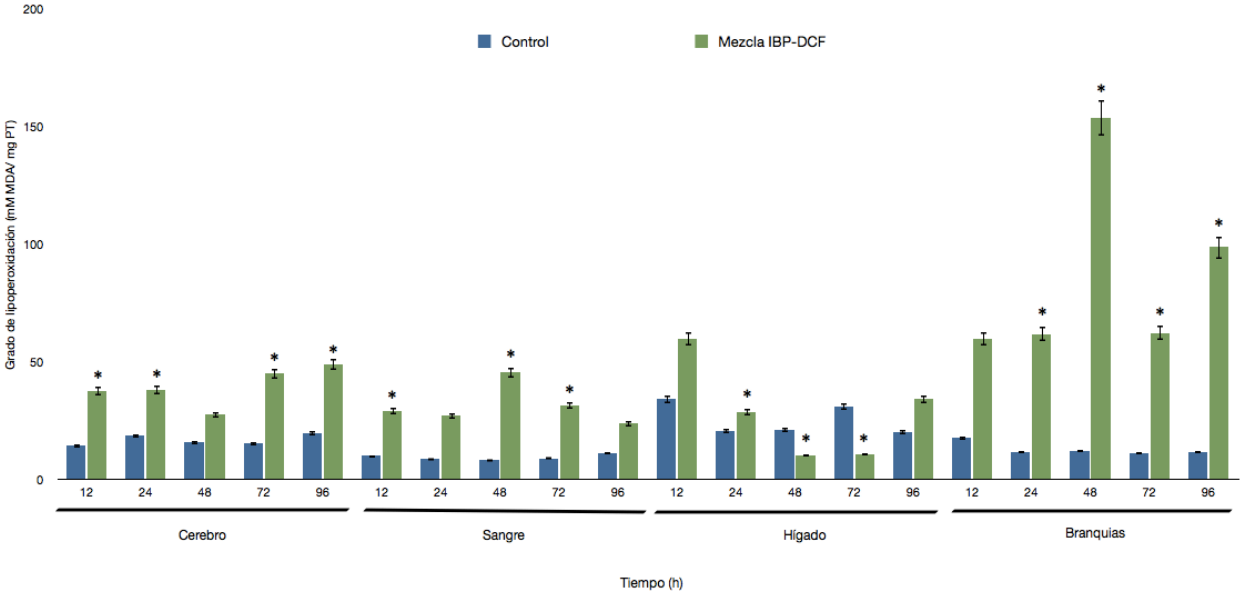

Figura 1. Grado de lipoperoxidación en cerebro, sangre, hígado y branquias de *C. carpio* expuesto a la mezcla IBP-DCF

- Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

8.1.2 Actividad de SOD

Para atenuar los efectos negativos de las EROs, los peces poseen un sistema de defensa antioxidante, como otros vertebrados. El antioxidante enzimático más importante es la SOD, encargada de catalizar la conversión del anión superóxido a peróxido de hidrógeno (Filho, 1996). En la figura 2 se muestra la actividad de SOD en los organismos expuestos a la mezcla IBP-DCF y en el grupo control, se puede observar que en el grupo expuesto a la mezcla se aumenta significativamente la actividad de SOD en el cerebro a las 12, 24, y 96 h con respecto al grupo control ($p < 0.05$), en sangre se muestra este aumento significativo a las 72 h y en hígado a las 24 h. Mientras que en el hígado a las 72 h se puede observar una disminución significativa de la actividad de esta enzima con respecto al grupo control ($p < 0.05$). Como se puede observar, en las branquias se presenta la mayor actividad de esta enzima ya que existe un incremento significativo a las 12, 24, 48, 72 y 96 h con respecto al grupo control ($p < 0.05$). Es posible que el incremento de la actividad de encontrado de esta enzima se deba a un mecanismo de defensa de las células a fin de contrarrestar el estrés oxidativo (Vlahogianni et al., 2007)

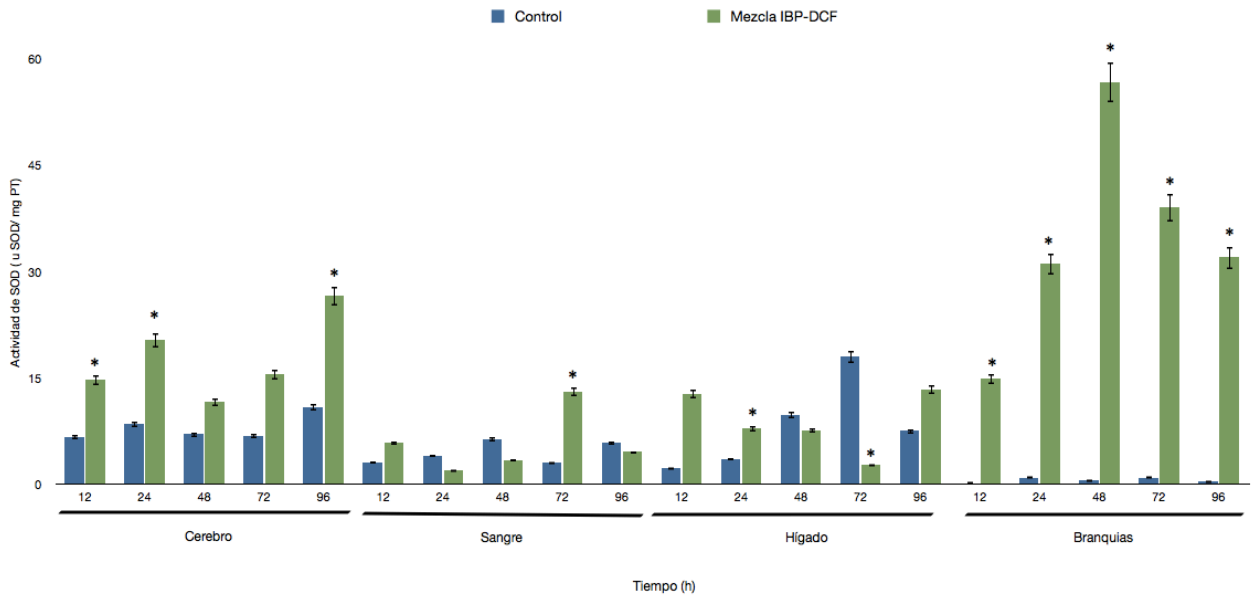

Figura 2. Actividad de SOD en cerebro, sangre, hígado y branquias de *C. carpio* expuesto a la mezcla IBP-DCF

- Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

8.1.3 Actividad de CAT

La CAT está asociada primariamente con peroxisomas en que se detoxifica el H_2O_2 llevándolo a dos moléculas de H_2O y O_2 (Hai et al., 1997). La actividad de CAT de los organismos expuestos a la mezcla IBP-DCF y el grupo control se muestra en la figura 3, de manera general se puede observar que hay una disminución de la actividad de esta enzima en los grupos expuestos respecto al grupo control, siendo en sangre a las 96 h una disminución significativa con respecto al grupo control ($p < 0.05$), mientras que en el hígado es el órgano que presenta la menor actividad al observarse a las 48 y 72 h una disminución significativa de la actividad de esta enzima con respecto al grupo control ($p < 0.05$). Este decremento puede deberse a que la sobreproducción de peróxido no solo activa a la CAT, sino que también favorece la oxidación y generación de metabolitos reactivos de los NSAID que tienen la capacidad de afectar la función de las proteínas.

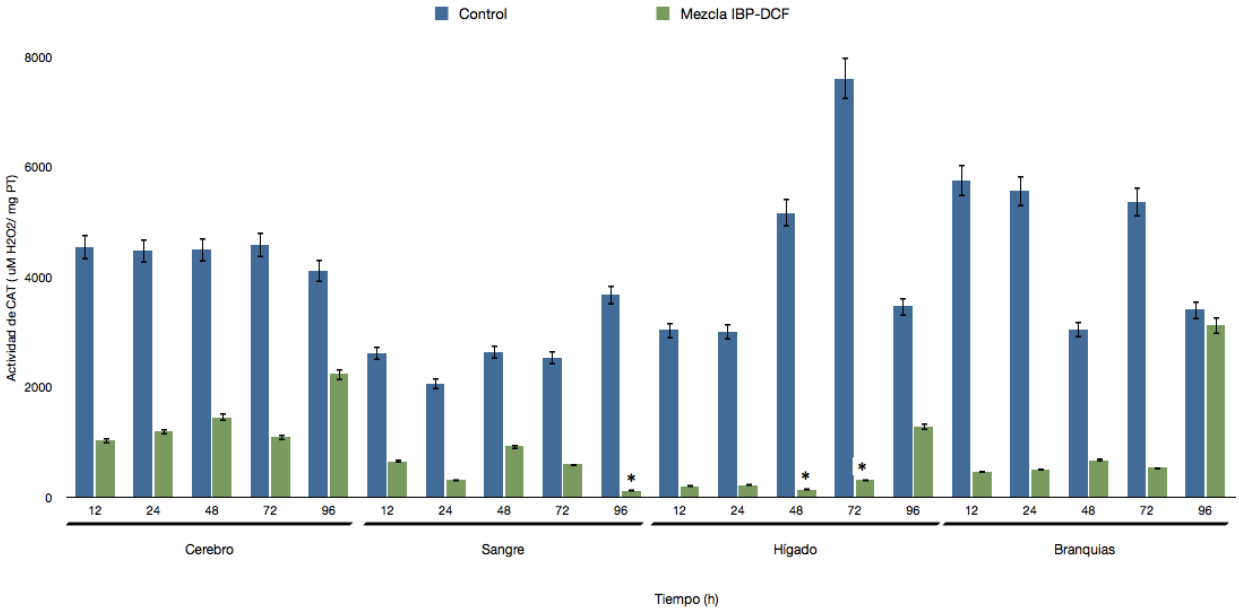

Figura 3. Actividad de CAT en cerebro, sangre, hígado y branquias de *C. carpio* expuesto a la mezcla IBP-DCF

* Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

8.1.4 Actividad de GPx

La Gpx es una enzima citosólica que reacciona con el H_2O_2 para reducirlo a H_2O y alcohol usando el GSH como agente reductor (Ahmad et al., 2000). Es la peroxidasa más importante que se ha postulado para proteger a los eritrocitos de los daños por H_2O_2 y los contaminantes ambientales pueden inducir la actividad esta enzima (Rajamanickam et al, 2009). En la figura 4 se muestra la actividad de GPx de los organismos expuestos a la mezcla IBP-DCF y el grupo control, se puede observar que hay una incremento significativo de la actividad de esta enzima en cerebro del grupo expuesto a las 12 y 72 h con respecto al grupo control ($p < 0.05$), en sangre a las 48 h, mientras que en hígado se observa un decremento significativo a las 48 h con respecto al grupo control ($p < 0.05$). Como se puede observar en la figura, las branquias son el órgano que presenta una mayor actividad de GPx, se muestra un incremento significativo a las 12, 24, 48, 72 y 96 h con respecto al grupo control ($p < 0.05$). Bagnyukova et al (2006) reportaron que los productos de LPX parecen estar involucrados en la regulación de las enzimas antioxidantes, por lo que el incremento del grado de LPX mostrado en este estudio podría explicar también el incremento de la actividad de esta enzima.

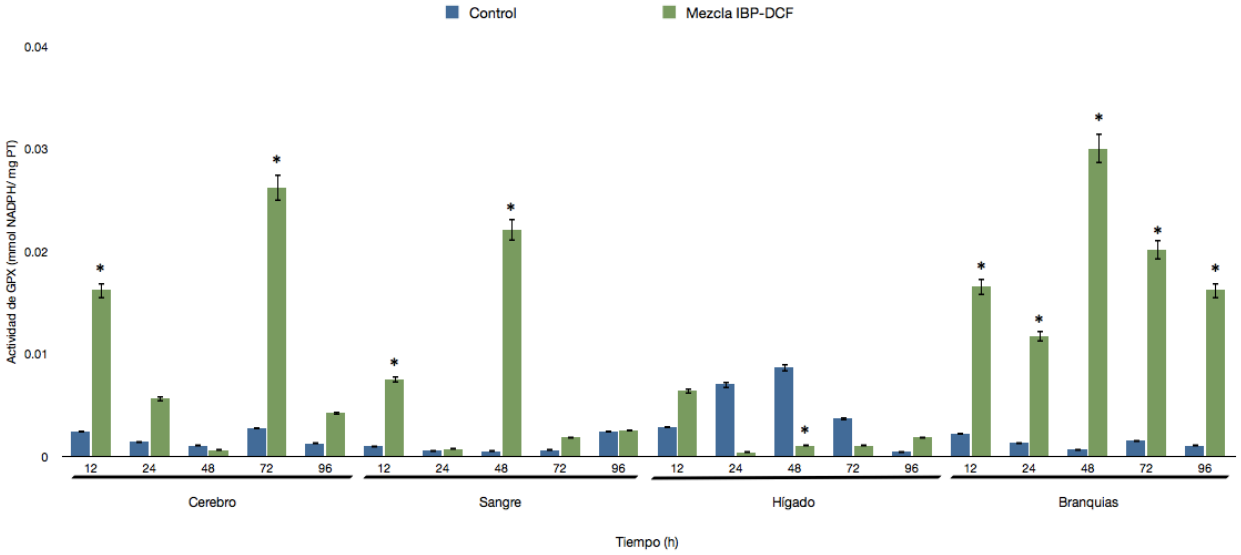

Figura 4. Actividad de GPx en cerebro, sangre, hígado y branquias de *C. carpio* expuesto a la mezcla IBP-DCF

* Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

8.2 Determinación del daño genotóxico a través de la prueba de micronúcleos

El origen de micronúcleos en eritrocitos de sangre periférica, se debe a la ocurrencia de lesiones en el DNA principalmente en las células en interfase-S, por ejemplo (1) roturas en los brazos cromosómicos que conducen a deleciones en el proceso anafásico, quedando rezagados fragmentos cromosómicos acéntricos con la consecuente formación de micronúcleos telómeros positivos, (2) roturas cromosómicas en los telómeros conducirían a la formación de puentes cromosómicos durante la anafase-telofase que al producirse roturas generan micronúcleos, (3) cambios en las secuencias nucleótidicas de DNA centromérico o en las secuencias génicas de proteínas centroméricas y durante la migración cromosómica originarían cromosomas rezagados y micronúcleos de cromosomas enteros con una o dos cromátidas, (4) alteraciones en las secuencias génicas de enzimas comprometidas con la condensación cromosómica produciendo cromosomas que no facilitan una segregación cromosómica equitativa que podrían formar micronúcleos o minicélulas.

En la figura 5 se muestra el recuento de micronúcleos por cada 1000 células de organismos expuestos por 24, 48, 72 y 96 h a IBP, DCF y a la mezcla IBP-DCF, además de un grupo control libre de fármacos. Como se puede observar la mezcla IBP-DCF produce incremento significativo del número de micronúcleos a las 24, 48 y 72 h con respecto al grupo control ($p < 0.05$), sin embargo el DCF es el produce el mayor número de micronúcleos a las 72 h con respecto al grupo control ($p < 0.05$). Este comportamiento se ha observado en otros estudios realizados en diferentes especies y con diferentes genotóxicos, en los que se reporta que durante los primeros días de exposición existen incrementos de micronúcleos y después de esos días los valores disminuyen. Estas variaciones en la disminución de MN después de una fase de incremento dependen del tipo de genotóxico, la concentración utilizada, la forma de administración y la respuesta genética que tenga cada especie (Cavas y Ergene-Gözülkara, 2003; Palhares y Grisolla, 2002; Grisolla y Torres-Cordeiro, 2000; Torres deLemos et al., 2007).

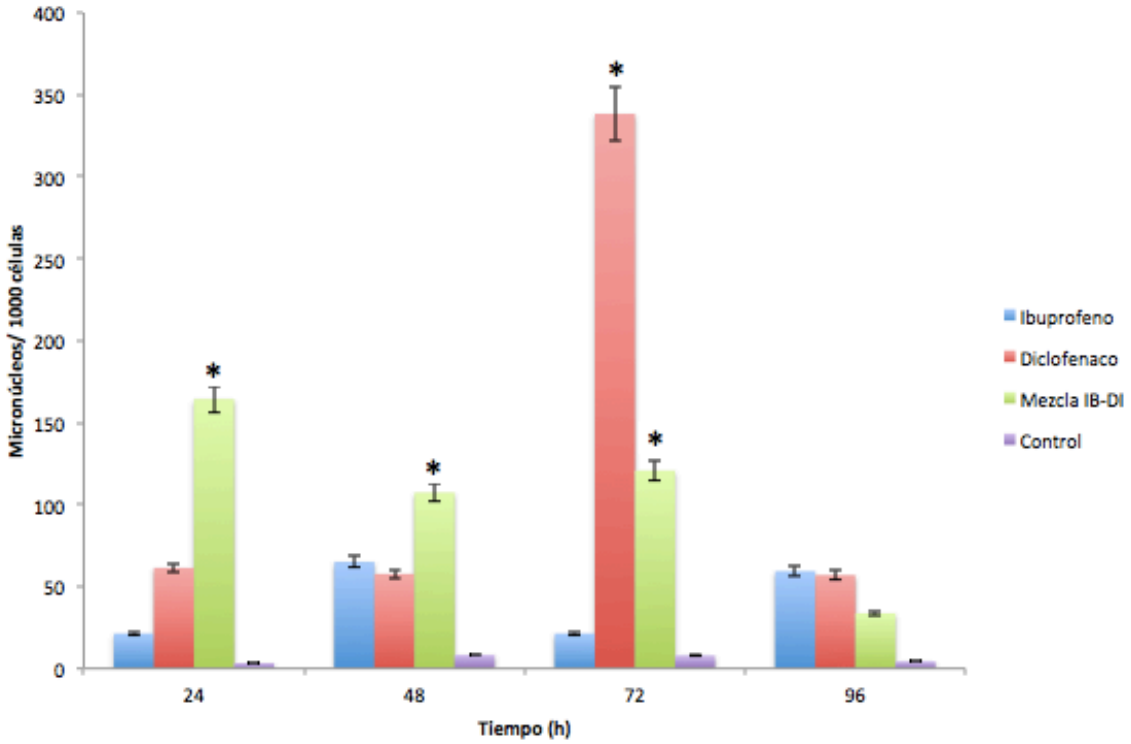

Figura 5. Recuento de micronúcleos en sangre de *C. carpio* expuesto a IBP, DCF y la mezcla IBP-DCF

- Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

En la molécula de ADN, los grupos nucleofílicos de la desoxirribosa y de las bases nitrogenadas quedan expuestos al ataque electrofílico de las especies reactivas del oxígeno (ERO), que llegan al interior del núcleo celular ya sean generadas como consecuencia de un agente externo, así como consecuencia de procesos metabólicos celulares (Shi et al., 1996). Existen diferentes tipos de daño oxidativo al ADN, entre los que se han reportado: ruptura del esqueleto azúcar fosfato de una o de las 2 hebras, modificación de las bases nitrogenadas (saturación y fragmentación del anillo de timina) y la formación de uniones cruzadas ADN-ADN ó ADN- proteína, a través de diferentes mecanismos: modificación de bases del ADN (la acción del radical $OH^{\cdot}$ da lugar a más de 20 modificaciones y entre ellas la más frecuente es la 8-hidroxi-2'-desoxiguanosina (8-OH-dG) que tiene un potencial altamente mutagénico al igual que la 5-hidroximetil-2-desoxiuridina), depurinación de bases del ADN (los sitios apurínicos o apirimidínicos se generan por ruptura del enlace glicosídico, que puede resultar del ataque al azúcar por parte del radical $OH^{\cdot}$) y rupturas de una cadena del ADN (se producen por escisión del enlace fosfodiéster, ocurren frecuentemente por ataque químico o de radicales libres a la porción desoxirribosa del esqueleto del ADN) (Reid et al., 1991). Se ha demostrado que la generación de EROs, provocan la aparición de rupturas de una cadena en el ADN, además de modificaciones de bases (Lo et al., 1996), lo que podría explicar el incremento de micronúcleos encontrados.

8.3 Determinación del daño citotóxico a través de la actividad de la caspasa 3

La citotoxicidad es una alteración de las funciones celulares básicas que con lleva a un daño que puede ser detectado. La apoptosis, o muerte programada de la célula, es un proceso activo (dependiente de energía en forma de ATP) caracterizado por la reducción de la célula en pequeños fragmentos (cuerpos apoptóticos) rodeados de una membrana plasmática intacta, la condensación del núcleo de la célula y la activación de caspasas, sin afectar a células vecinas. Existen dos rutas principales que conducen a la muerte por apoptosis. La vía extrínseca, iniciada por la unión de ligandos específicos a ciertos receptores situados en la membrana que rodea la célula, lo que produce la activación de la caspasa-8, la cual comienza la cascada de activación de otras moléculas que conducen a la muerte celular. La otra vía es la intrínseca, iniciada a nivel mitocondrial, con la ruptura de la membrana que rodea la mitocondria y formación de poros, a través de los cuales se produce la liberación de factores (citocromo c) que activan la caspasa-9,

que consecuentemente inicia la cascada que conduce a la muerte por apoptosis. En este estudio la actividad específica de la caspasa 3 de *C. carpio* expuesto por 24, 48, 72 y 96 h a IBP, DCF, a la mezcla IBP-DCF y un grupo control libre de fármacos, se muestra en la figura 6. Se observa que solo el IBP a las 24 h produce un incremento significativo con respecto al grupo control *($p < 0.05$)*.

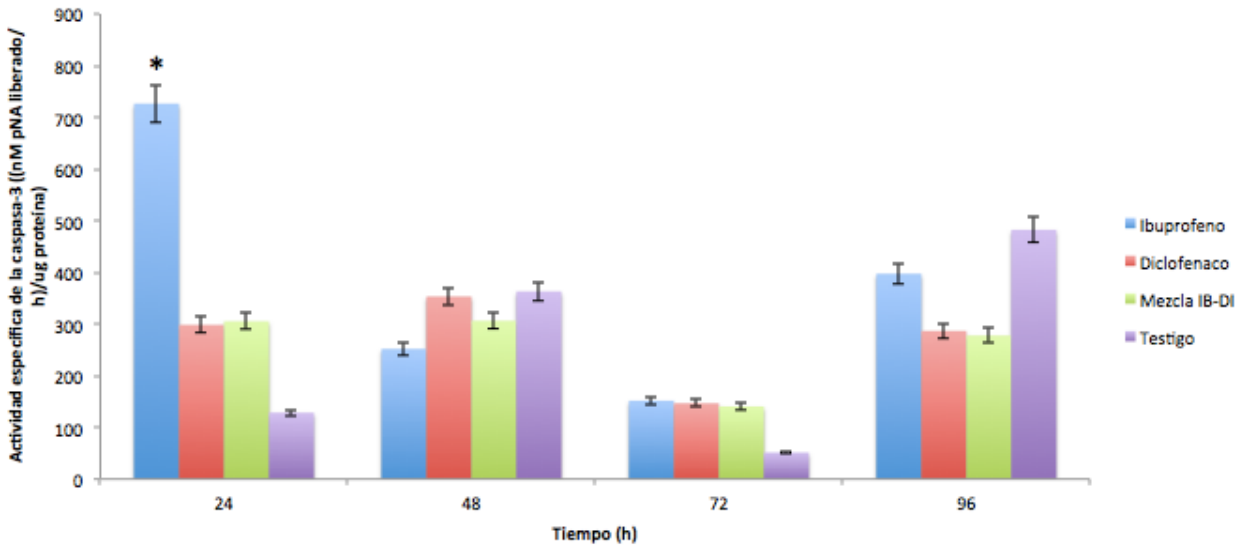

Figura 6. Actividad específica de la caspasa-3 en sangre de *C. carpio* expuesto a IBP, DCF y la mezcla IBP-DCF

* Indica diferencia estadísticamente significativa respecto al grupo control ($p < 0.05$)

Una gran cantidad de estudios indican que el incremento en la generación de EROs, y un déficit en las defensas antioxidantes, así como la disminución en la eficiencia de los mecanismos de reparación del DNA y la proteólisis, además de pérdida de regulación del sistema inmune, son factores que contribuyen primariamente al aumento de estrés oxidativo (Olanow y Arendash, 1994), además las modificaciones en las proteínas, como carbonilación, nitración y unión cruzada proteína-proteína, se asocian por lo general con pérdida de función y pueden llevar por un lado al desdoblamiento y degradación de las proteínas dañadas, o por el otro, a su agregación, resultando en acumulación como inclusiones citoplásmicas, y a la muerte celular (Dalle-Donne et al., 2005). Cualquier clase de estímulo estresante, tal como exposición a EROs, daño al ADN o incremento del calcio extracelular generado por la inhibición de las prostaglandinas, puede iniciar la vía

intrínseca de la apoptosis donde se induce un cambio en la permeabilidad de la membrana mitocondrial, lo cual lleva a edema y a una declinación del potencial de membrana mitocondrial, se libera el citocromo c al citoplasma, el cual se une a la Apaf-1 (proteína factor 1 activadora de la proteasa apoptótica) que utilizando ATP/ADP activa a la caspasa 9, la cual a su vez activa a la caspasa efectora 3, la cual hidroliza sustratos específicos que eventualmente lleva a la muerte celular, lo que podría explicar el aumento del IBP. El decremento en los tiempos posteriores puede deberse a que en la célula también se cuentan con proteínas antiapoptóticas (bcl-2), proteínas inhibidoras de la apoptosis (AIP) y proteínas de choque térmico (Hsps), las cuales actúan a diferentes niveles de la cascada de la apoptosis, los cuales se muestran en la figura 7.

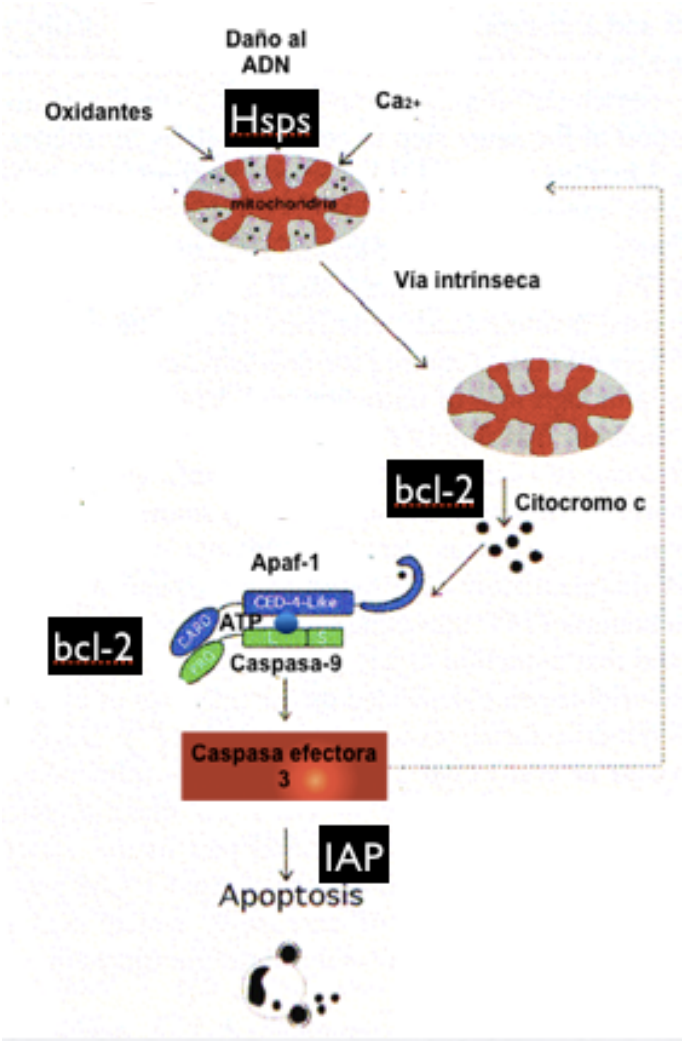

Figura 7. Vía intrínseca de la apoptosis

8.4 Análisis isobolográfico

La toxicidad en los ecosistemas naturales por lo general no resulta de la exposición a una sola sustancia, sino que es resultado de la exposición a mezclas de sustancias tóxicas (Altenburger et al, 1996; Backhaus et al, 2003). Una forma de determinar si la toxicidad de una mezcla binaria se debe a una interacción (antagonista o sinérgica) es llevar a cabo experimentos basados en el método isobolográfico (Berenbaum, 1989). El modelo se basa en la ecuación:

$$\left(\frac{d_A}{D_A}\right)^{1/\lambda} + \left(\frac{d_B}{D_B}\right)^{1/\lambda} = 1$$

en la que la ecuación isobolográfica estándar se amplía con un parámetro adicional (λ), dA y dB representan la concentración de la sustancia tóxica A y B aplicadas en la mezcla a un nivel determinado de efecto; DA y DB representan las concentraciones de las sustancias tóxicas A y B que son necesarias para obtener el mismo nivel de efecto cuando se aplican solos. Ambas sustancias se aplican en concentraciones isoefectivas. El parámetro λ refleja el grado de sinergia / antagonismo. Si $\lambda<1$ el efecto es antagónico, si $\lambda = 1$, el efecto es aditivo y si $\lambda >1$ el efecto es sinérgico. (Sørensen et al., 2007). Los datos experimentales de toxicidad de mezclas son limitados. La información disponible sugiere que la adición de la concentración, en forma exacta o aproximada, es bastante común, y en cualquier caso es un modo de referencia importante de la acción. Y sería posible de prever cuando los tóxicos actúan sobre los sistemas biológicos similares o idénticos, y cuando los mecanismos de acción están completamente correlacionados. Se sugiere que el antagonismo puede esperarse cuando los tóxicos actúan en diferentes sistemas biológicos y cuando no están correlacionados los mecanismos de acción (Anderson y Weber, 1975). En la tabla 4 se muestran los resultados obtenidos del análisis isobolográfico de *Cyprinus carpio* expuestos por 96 h a la mezcla IBP-DCF, en esta tabla se presenta el valor de I obtenido con la fórmula del análisis y el tipo de interacción toxodinámica que produce esta mezcla con los diferentes biomarcadores utilizados. De manera general se puede observar que el tipo de interacción toxodinámica que predomina en la mezcla IBP-DCF y con los biomarcadores utilizados es de antagonismo, la excepción es la actividad de la CAT en la que se muestra una interacción de tipo sinérgico en sangre, cerebro, branquias e hígado de *Cyprinus carpio*.

Tabla 4. Valor de I y tipo de interacción de la mezcla IBP-DCF en *C. carpio*

Biomarcador	Órgano							
	SANGRE		**HÍGADO**		**CEREBRO**		**BRANQUIAS**	
	Valor I	***Tipo de interacción***	***Valor I***	***Tipo de interacción***	***Valor I***	***Tipo de interacción***	***Valor I***	***Tipo de interacción***
LPX	6.17	A	3.75	A	11.12	A	13.32	A
SOD	1.93	A	5.51	A	7.73	A	7.13	A
CAT	0.08	S	0.31	S	0.82	S	0.83	S
GPx	17.43	A	1.79	A	13.03	A	44.26	A
Actividad específica de la caspasa-3	1.67	A						
Recuento de micronúcleos	1.16	A						

A= Antagonismo, S = Sinergismo

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