Water contamination by delorazepam induces epigenetic defects in the embryos of the clawed frog Xenopus laevis

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Water contamination by delorazepam induces epigenetic defects and genomic instability in the embryos of the clawed frog *Xenopus laevis*

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Abstract

Delorazepam, a derivative of diazepam, is a psychotropic drug belonging to the benzodiazepine class. Used as a nervous-system inhibitor, it treats anxiety, insomnia, and epilepsy, but is also associated with misuse and abuse. Nowadays benzodiazepines are considered emerging pollutants: conventional wastewater treatment plants indeed are unable to eliminate these compounds. Consequently, they persist in the environment and bioaccumulate in *non-target* aquatic organisms with consequences still not fully clear. To collect more information, we investigated the possible epigenetic activity of delorazepam, at three concentrations (environmental, 1 µg/L; and near future, 5 and 10 µg/L) using *Xenopus laevis* embryos as a model. Analyses demonstrated a significant increase in genomic DNA methylation and differential methylation of the promotors of some early developmental genes (*otx2, sox3, sox9, pax6, rax1, foxf1* and *myod1)*. Moreover, studies on gene expression highlighted an unbalancing in apoptosis/proliferation pathways, suggestive of genomic instability, given by the aberrant expression of DNA-repair genes. Results are alarming considering the growing trend of benzodiazepine concentrations in superficial waters, especially after the peak occurred as a consequence of the pandemic COVID-19, and the fact that benzodiazepine GABA-A receptors are highly conserved and present in all aquatic organisms.

Introduction

Benzodiazepines (BZDs) are the most used psychotropic drugs for the treatment of anxiety and insomnia. It has been estimated that they are used by about 2–7.5% of the general population and, among these, more than half are long-term users (>3 months) (Lugoboni et al., 2018). Dependence, in fact, develops in nearly half of the patients who use them for at least 1 month. The harm deriving from their assumption is still underestimated: wide disparities are present between clinical indications for rational use and their real-life use. The tendency to abuse and misuse these drugs (Votaw et al., 2019; Shah et al., 2021) has increased in the last two years: COVID-19 and the ongoing war caused a spike in depression, insomnia, and post-traumatic stress disorders, and a consequent dramatical increase in BZDs consumption (Sarangi et al., 2021; Mattiuzzi et al., 2022), also by that part of the population who generally had never used them before.

After the assumption of the active principle, unmetabolized molecules and their metabolites are excreted into sewage treatment plants, whose inefficiency in the total elimination of these compounds is well known (Patel et al., 2019). Benzodiazepines are exponentially detected in most sewage, surface, and coastal waters, of all industrialized countries, especially in hospital effluents (Cunha et al., 2019; Pereira et al., 2020) at concentrations ranging from nanogram to microgram per liter (Hernando et al., 2006). Accumulation induces adverse effects on living organisms, especially in freshwater habitats, due to bioaccumulation, behavioral changes and modulations in genes expression and enzymes (Nunes et al., 2019; Fogliano et al., 2022a; Fogliano et al., 2022b). Anyway, we are still unaware of how these trace levels in the environment can indirectly affect humans.

Increasing evidence shows that the epigenotoxic effects of emerging pollutants, represented by DNA methylation and demethylation, are significant in the assessment of water safety (Hu and Yu, 2019). It is known that exposure to environmental contaminants such as persistent organic pollutants, volatile organic compounds, endocrine-disrupting chemicals, and heavy metals can trigger unintentional alterations in DNA methylation in cells and living organisms *in vivo* (Hu and Yu, 2019; Burgio et al., 2018; Hou et al., 2012). Therefore, DNA methylation patterns, including global and gene-specific DNA methylation, are being widely applied to explore the epigenetic responses of living organisms to external environmental conditions and pollution. The effects of pharmaceuticals have been largely neglected so far although it is well-known that they can exert persistent epigenetic changes in patients (Csoka and Szyf, 2009; Toth, 2021). Benzodiazepines, in particular, are designed to affect cell pathways modulating signaling and gene expression (Seo et al., 2014), and chromatin remodeling is already a proven pharmacological effect (Csoka and Szyf, 2009). Excessive DNA methylation leads to transcription silencing and gene inactivation, while reduced DNA methylation results in chromosomal instability (Eden et al., 2003; Kandi and Vadakedath, 2015).

Xenopus laevis is commonly used in ecotoxicological studies and, in a previous study, we reported an embryotoxic activity of delorazepam, which profoundly influenced early development by inducing mortality and malformation, together with increased oxidative stress and altered gene expression of early developmental genes (Fogliano et al., 2022a). In literature is reported that oxidative stress, in particular, seems to be directly involved in the epigenetic regulation of gene expression, specifically in controlling DNA methylation (Franco et al., 2008; Ding et al., 2019)

Therefore, in this study, we verified the potential epigenotoxic effect of the long-acting delorazepam (DLZ), benzodiazepine derivative of diazepam, on *Xenopus laevis* embryos after exposure to three environmentally relevant contamination levels. In particular, we first assessed global DNA methylation and, more specifically, also methylation pattern of the promoters of some master genes

involved in early embryonic development (*otx2, sox3, sox9 pax6, rax1, foxf1* and *myod1*). In addition, considering that methylation defects contribute to chromosomal imbalances (Jones & Gonzalgo, 1997; Kandi and Vadakedath, 2015), we also paid attention to the expression of DNA repair genes (*gadd, rad51, mutL*) and genes involved in proliferation/apoptotic pathways (*p53, bax, c-jun,c-fos,*). Finally, based on the evidence that DLZ induces marked developmental defects (Fogliano et al., 2022a), we started a specific study to determine the level of methylation in the head, intestine, and tail tissues.

2. Materials and Methods

2.1 Animals

Adult *Xenopus laevis*, obtained from Nasco (Fort Atkinson, Wisconsin, USA), were kept and used at the Department of Biology of the University of Naples, Federico II, according to the guidelines and policies dictated by the University Animal Welfare Office in agreement with international rules and strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the Italian Ministry of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II (Permit Number: 2014/0017970). All procedures were performed according to Italian ministerial authorization (DL 116/92) and European regulations on the protection of animals employed for experimental and other scientific purposes. All surgical procedures were performed under tricaine (MS222, Sigma) and organized to minimize suffering. Fertilized eggs and embryos were obtained as described in Fogliano et al. (2022a). Embryo staging was according to Nieuwkoop and Faber (1956).

2.2 Embryos' treatment

As in Fogliano et al., 2022, three *in vitro* fertilizations were performed. For the treatments, a largely consumed pharmaceutical, containing the active principle delorazepam at a 1 mg/ml concentration, was diluted in FETAX solution in order to obtain a final concentration of 1 µg/L, 5 µg/L and 10 µg/L. Sibling embryos grown in FETAX solution were used as controls. The pH (7.4) of the solutions was checked daily. All embryos were exposed up to stage 45/46 in a static condition: solutions were not renewed, so as to determine the potentially embryotoxic effects of the active principle and/or its active metabolites. All the experiments were carried out at 21 °C, under a 12 h light: 12 h dark photoperiod.

2.3 DNA extraction

For DNA extraction, a pool of 10 embryos at stage 45/46 for each treatment/fertilization was used and performed according to the protocol described by Marques et al. (2017). Briefly, the embryo

pellets were overlaid with 100 µL of lysis solution (10 mM Tris–HCl, 25 mM ethylene diamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 75 mM NaCl) in the presence of 0.2 mg/mL proteinase K, 50 mM DTT and 0.5 µg glycogen (Invitrogen, Cergy-Pontoise, France) and incubated overnight at 50 °C. DNA was isolated by standard phenol-chloroform extraction.

2.4 Global Methylation Level Luminometric Methylation Assay

Luminometric Methylation Assay (LUMA), a pyrosequencing-based method (Johansson et al., 2006), was used to test the global methylation level of each DNA sample, as previously described (Congras et al. 2014). 500 ng of genomic DNA was digested by EcoRI-HF and by either HpaII or MspI (New England Biolabs, France). Enzymatic digestion of DNA was performed using 10 units of restriction enzymes and 4h of incubation to guarantee the efficiency of the reaction. Digestion efficiencies were checked on an agarose gel. Each digested DNA sample was then diluted in Pyromark Annealing Buffer (Qiagen, Hilden, Germany) and then pyrosequenced on a PyroMark Q24 sequencer (Qiagen; product no. 9001514) using PyroMark Gold Q24 Reagents (Qiagen; product no. 970802). The isoschizomers HpaII and MspI target the same DNA CCGG sequence. The peak height of C + G incorporation was normalized by the peak height of A + T incorporation to normalize for digestion efficiencies. The calculated ratio between the peak height of simultaneous C + G incorporations in HpaII and MspI digests is, therefore, representative of the DNA methylation level of the sample. The ratio is close to 1 when the sample is fully unmethylated.

2.5 Analysis of Promoter Region Methylation by Bisulfite Conversion and Pyrosequencing

Bisulfite conversion using 1 µg of control and treated DNA was performed using the EpiTect Bisulfite Kit (Qiagen, product no. 59104) following the manufacturer's instructions. Primer pairs designed by the PyroMark AssayDesign 2.0 software (Qiagen; product no. 9019062) on our sequences of interest (Table I) were used to amplify converted DNA using the PyroMark PCR kit (Qiagen; product no. 978703). We used 1 µl of bisulfite-converted DNA (50 ng) in 25 µl reaction volume, according to the manufacturer's instructions. The PCR program was: 3 min at 94°C followed by 45 cycles of 30 sec at 94 °C, 1 min at 60 °C for hybridization and 1 min at 72 °C for elongation, with a final extension of 10 min at 72°C. Amplicons were then pyrosequenced on a PyroMark Q48 Autoprep pyrosequencer (Qiagen; product no. 9002471 with the appropriate buffers and solutions (Qiagen; product no. 974022)). The reverse primers were biotinylated for all amplified regions in order to allow the PCR product purification by binding on Streptavidin-coated sepharose magnetic beads (Qiagen, 974203). Each analyzed region was assayed on biological triplicates. The methylation percentage per CpG was then obtained by calculating the mean of all replicates that passed the control quality of the Pyromark

Q48 software. The average methylation percentage of all CpGs analyzed in a given region was computed for each individual.

Gene	Oligo sequence	Position from the TSS (+1)
otx2 FOR	GAGGAAAAGAAAGGTTGAATTGTAG	-332
otx2 REV	ATATACAACTATAAACTCCACACCA	-230
otx2 Seq	GAAAGGTTGAATTGTAGTT	
sox3 FOR	TTGAATAGTGGAAGTGGAAAAGTTAAT	-253
sox3REV	ACCCTCCTCCCCCCTAAACAAATATAAT	-200
sox3 Seq	GTAGGGAGGATTTAATTTTT	
sox9 FOR	GGAAGGGTATAAAAAGGGTATTGATT	-635
sox9 REV	AAAACTAAACATTCATCTCCAATACAT	-545
sox9 Seq	TTTAATGGTAGATATTGTTTTGAT	
pax6 FOR	GTAGAATGAGGTTGGAAATAAGGTAGA	-480
pax6 REV	TTCCTCTCCCCTCTTCAATACTAA	-400
pax6 Seq	AGAGAGAGAGATATTTTTTTG	
rax1 FOR	AGTTGTGGAAGTTTTGAAATATTTG	-705
rax1 REV	TAACCCATTACATTAAATTTCATACACTTC	-650
rax1 Seq	GTTTTTAATAGTAAAGTGTTTAAGT	
foxf1 FOR	TGGTTAAGGGGGAGTGTTT	-220
foxf1 REV	ACTACTCCACCTCCAACTTCTAAATTATAC	-70
foxf1 Seq	AGTAGAAGGGGGGTT	
myod1 FOR	ATTAATTAGGGAATTAGTATTTGGTTTTG	-740
myod1 REV	AACCTCTTCTACCCCAAATAT	-670
myod1 Seq	ATTTGGTTTTGATTTTTTAGTAATA	

Table 1. Primers used on sequences of genes of interest

2.4 RNA isolation and gene expression quantification

Total RNA was extracted from a pool of 6 whole embryos from each treatment/fertilization with the Direct-zol RNA Mini Prep kit (ZymoResearch, Irvine, CA, USA) and used for cDNA synthesis using the SuperScript Vilo cDNA synthesis kits (Life Technologies Massachusetts, USA). Primers used are listed in Table 2. Real-time PCR was performed using Power SYBR Green Master Mix kits (Life Technologies) using the 96-well optical reaction plate in 20 μL total reaction volume. Reactions were conducted on an AriaMx Real-time PCR System. β-actin was adopted as the endogenous control. The magnitude of change in gene expression was determined by the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001).

Gene	Oligo sequence
actin *for* actin *rev*	ATTGAGCCACCAATCCAGG ACAAGTGTTGGAATGTGCG
c-jun *for* c-jun *rev*	TGTGTGCGGGAAGTCCT ACCTAGTGTCTCTAGCAGTTTAACC
c-fos *for* c-fos *rev*	CTCATGCCGGTGCTGG CTGGGTATGTGAAGACAAATGATGTT
p53 *for* p53 *rev*	GGTTCGAGTGTGTGCCTG CGCCCCTTAATCCGCAA
bax *for* bax *rev*	AAGCAAGGGGAAGGCG CGGGTCACTCTGTTGGG
gadd *for* gadd *rev*	AAGACAATCAGGATGTCGCT CCTTGATCTGTAATTCATGTGGGT
rad51 *for* rad51 *rev*	ATTCCTATGCAATGGCCGT GGCGCTTGCCTCCAGTAA
mutL *for* mutL *rev*	TATGAGGACTGAGAAAACAGAAGCA AGTTACAAACACTTGGAGCTGAT

Table 2. List of primers used to perform the Real-Time PCR

2.5 Statistical Analysis

For global methylation, differences in global methylation levels were assessed by a *t-test*. For promoter methylation, groups were compared using a permutation test for k-independent samples (Monte-Carlo sampling of 100,000 permutations, coin R package) followed by pairwise comparisons (1000 permutations; Benjamini–Hochberg correction).

3. Results

3.1 Effect on global DNA methylation

The mean global DNA methylation showed a significant, dose-dependent, increase from 44.7% in controls, to 47.2% in embryos treated with 1 µg/L, to 52.8% and 55.3% respectively for embryos treated with the two higher concentrations ($p<0.001$; $p<0.0001$).

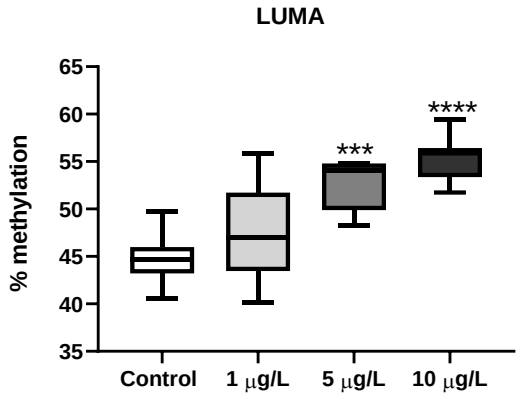

Figure 1. DNA global methylation level of *Xenopus laevis* embryos treated with delorazepam (1 µg/L; 5 µg/L; 10 µg/L). Determination by Luminometric Methylation Assay (LUMA). ***p<0.001; ****p<0.0001.

3.2 Effects on the methylation status of the promoter region of early development genes

The analysis covered from 2 to 8 CpGs of the promoter of each gene. Most CpGs of *otx2, pax6, foxf1* and *myod1* resulted significantly less methylated in embryos exposed to 5 or 10 µg/L, with respect to promoters of controls and embryos exposed to 1 µg/L (fig. 2). In contrast, all treated embryos displayed hypermethylation of CpGs located on the promoters of *sox3, sox9* and *rax1*.

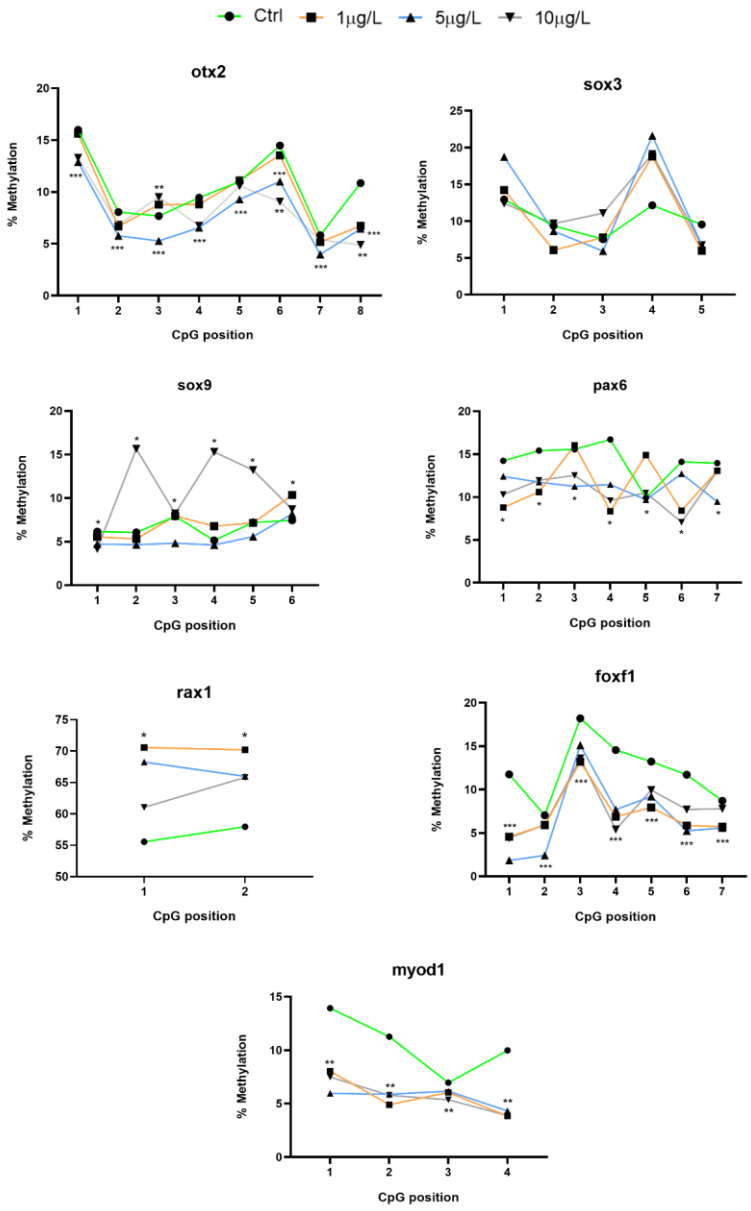

Figure 2. Methylation pattern in the promoters of early developmental genes of *Xenopus laevis* embryos exposed to increasing doses of delorazepam (1 µg/L; 5 µg/L; 10 µg/L). *p< 0.05; **p<0.01; ***p<0.001.

3.4 Proliferation and genomic instability

For *p53*, treatment with DLZ induces significant overexpression at the environmental concentration, higher at the intermediate concentration ($p<0.0001$), with a significant decrease at the higher concentration if compared to 5 µg/L and control ($p <0.05$). A similar trend is also observed for *bax*, even if a significant increase is noted only starting from the intermediate concentration ($p <0.0001$), also in this case level decrease with the higher concentration, but still resulting overexpressed if compared to the control ($p<0.01$). For *c-jun* and *c-fos* on the contrary, an exponential increase in expression levels is observed, without decrement, in particular at 5 µg/L and 10 µg/L treatment ($p <0.0001$).

Regarding DNA repair genes, the situation is variable. For *gadd* and *rad51*, a general downregulation is observed, with very significant decrement at the two higher concentrations ($p<0.0001$) for *gadd*. *mutL* tends to be slightly over-expressed at the first two concentrations, with a decrease at the highest concentration.

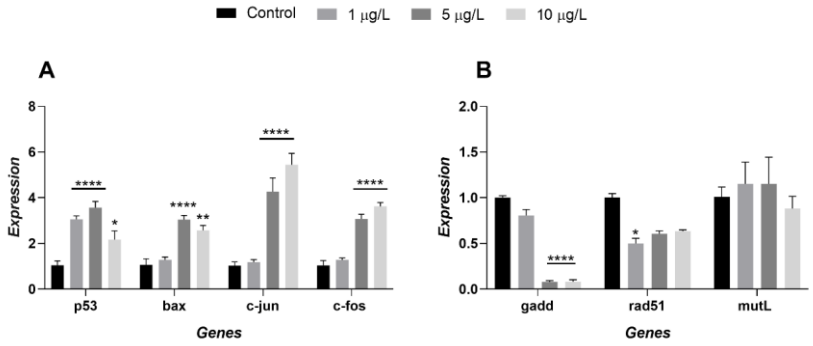

Figure 3. Changes in the expression of genes involved in (A) apoptotic/proliferation pathways and (B) DNA repair mechanism, in *Xenopus laevis* embryos exposed to increasing doses of delorazepam (1 µg/L; 5 µg/L; 10 µg/L). *p53* (5 µg/L vs 10 µg/L = *$p<0.05$). Data are means ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Discussion

Growing evidence suggests that the epigenome (Burgio et al., 2018; Ryu et al., 2015; Santangeli et al., 2016) fluctuate in response to environmental stressors (Vaudin et al., 2022), but the role exerted by pharmaceutical pollutants, and, in particular, by psychotropic drugs is still rather unknown. This is a quite surprising circumstance if it is considered that their pharmacological efficacy is linked to their epigenetic activities (Csoka and Szyf, 2009; Boyadjieva & Varadinova, 2012).

DNA methylation is the best-known DNA epigenetic modification (Moore et al., 2013). It has been associated with various diseases (Van Vliet et al., 2007), altered development (Anway et Skinner, 2008), and environmental stress (Bollati et al., 2008), including exposure to contaminants (Vaudin et al., 2022). Embryogenesis, in fact, is an extremely critical period (Wilkinson et al., 2016), in which the DNA methylation pattern is susceptible to various interferences, with consequent disturb to cell differentiation and organogenesis, causing teratogenesis (Szyf, 2011) or even death. In our recent study (Fogliano et al., 2022a) delorazepam was proven to induce a dose-dependent increase in embryo malformation and mortality. In the same study, changes in gene expression were also demonstrated, thus prompting the present study in which epigenotoxic effects were investigated.

Commenté [HA1]: https://doi.org/10.1038/npp.2012.112

Results obtained confirm the hypothesis and reveal that significant hypermethylation of total DNA occurs in *Xenopus* embryos exposed to DLZ. In general, a correct DNA methylation pattern is essential for normal vertebrate development, therefore global demethylation and remethylation occur at specific stages during embryo development (Yan et al. 2022). In *Xenopus*, DNA methylation has a role in regulating the timing of gene activation at the midblastula transition (MBT): the high levels of methylation in the DNA of both oocyte and sperm are maintained in the early embryo but progressively decline during the cleavage stages. As a result, the *Xenopus* genome has its lowest methylation content at MBT and during subsequent gastrulation (Stancheva et al., 2002). The global DNA hypermethylation, established as a result of the influence of the DLZ, would disturb the typical fine regulation of embryonic development, a time in which the different embryonic lineages are progressively and independently methylated to drive the correct differential development. An increase of DNA methylation in humans was already observed in response of environmental pollutant such as organic pollutants and dioxin (McClure et al., 2011; Lind et al., 2013) and even associated with increased suicide attempts in psychiatric patients (Murphy et al., 2013).

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Given that methylation events affect the entire genome, particular attention has been dedicated to methylation of promoters, one of the foremost mechanisms responsible for cell differentiation during embryogenesis, working by excluding from transcription the unwanted genes (Oligny, 2001).

Investigations showed that *otx2, sox3, sox9, pax6,* and *rax1*, genes involved in nervous system differentiation, presented CpGs significantly less or more methylated, endorsing the previously raised hypothesis of interference of DLZ on the nervous system development. In particular, the *otx2* gene encodes a transcription factor essential for the normal development of the brain, cerebellum, and pineal gland and it is required for the neurogenesis of dopaminergic neurons in mesencephalon, also acting in the very first steps of ocular embryogenesis. In humans, OTX2 mutations are associated with microphthalmia and brain abnormalities (Beby & Lamonerie 2013). Analysis of the *otx2* promoter revealed many CpG sites that were significantly less methylated than the control, especially at higher DLZ concentrations, thus indicating less controlled expression. *sox3,* required for the initiation of neurogenesis and with a pivotal role in the establishment and subsequent development of the CNS, presented as well CpGs with differential methylation, mainly hypermethylated, compared to the control. A loss of *sox3* expression is a critical event in eye formation (Dee et al., 2008). *sox9, pax6,* and *rax1*, genes controlling neurulation and with a central role in eye and retina formation, also displayed a misregulation of their promoter, represented by hypo and hypermethylated CpGs if compared to control, therefore confirming the altered gene expression previously highlighted (Fogliano et al., 2022).

Although there is no direct correlation between the degree of the CpGs methylation of the promoters and what will be the effective gene expression (Siegfried & Simon, 2010), what seems certain is the alteration and/or misregulation of these fundamental genes during the very early stages of development. These epigenetic alterations could be at the origin of the abnormal expression of these genes and support the teratogenic effect of DLZ explaining the increase of malformations e/o mortality observed in treated embryos (Fogliano et al., 2022).

Further support to this hypothesis is the strong hypomethylation of all CpGs in the promoters of *myod1* and *foxf1* genes, respectively implicated in the regulation of skeletal myogenesis and gut development: among the most evident morphological alteration, there was an incorrect development of the gut and abnormal tail conformation.

One of the possible explanations for the altered methylation pattern could be also found in the elevated ROS levels, previously shown to be dose-dependent increased in DLZ-treated embryos (Fogliano et al., 2022). ROS production is, in fact, associated with alterations in DNA methylation patterns, where once the changes in the ROS levels exceed the ability of cellular defense, abnormal hypo and hypermethilation occur (Donkena et al., 2010; Hu et al., 2021).

It is known that methylation defects contribute to chromosomal imbalances and DNA instability. The phenotypic effects induced by long-lasting alterations in gene expression, in fact, can considerably

promote cell cycle disorders, cell transformation, and stress-induced apoptosis (Lappalainen and Greally, 2017; Arechederra et al., 2018). Real-time PCR of *Xenopus* embryos treated with DLZ revealed modified expression of *gadd*, *rad51*, *mutL*, genes involved in DNA repair. In particular, *gadd*, *rad51* and, to a lesser extent, *mutL*, were strongly downregulated, suggesting a negative effect on DNA repair mechanisms. Dysregulated DNA damage response can result in a number of cell fate possibilities, including continued proliferation under replicative stress associated with loss of cell cycle checkpoint regulation, increased genome instability, increased chromosome aberrations, mitotic slippage and aneuploidy. As a consequence of the loss of genome stability, the accumulation of mutations can induce a malignant phenotype (Hoeijmakers, 2001). *gadd* gene is crucial for DNA damage repair and its abnormal expression was related to unusual cell proliferation (Liu et al., 2015; Peng et al., 2015; Zhang et al., 2020), while the attenuation of expression of *rad51* and *mutL*, which plays a critical role in mismatch repair, was as well observed in many tumor cells (Javid et al., 2018; Bonilla et al., 2020).

Moreover, in DLZ-treated embryos, the master tumor suppressor gene *p53*, involved in arresting the cell cycle after DNA damage, and the pro-apoptotic *bax* (Chen et al., 2014) were significantly upregulated at 1 µg/L and 5 µg/L concentrations. This is probably indicative of an attempt made by the embryo to attenuate the damage by eliminating damaged cells. The hypothesis is supported by the down-regulation of *gadd* and *rad51*, DNA repair genes. Classically, cells with damaged DNA are expected to arrest at the G2 checkpoint, and, if damage is not repaired, they are supposed to initiate apoptosis (Cejka et al., 2003). The hypotesis of possible attempt to reaction is endorsed by paradoxical expression of *abcb1* gene, involved in detoxifying mechanisms, overexpressed at the same DLZ concentrations (Fogliano et al., 2022).

The reaction and defense mechanism probably actuated at lower concentrations is no longer effective to counteract the negative outcomes at the higher concentration of 10 µg/L, where a contemporary downregulation of the proapoptotic genes and DNA repair genes occurs (as well as *abcb1* levels drastically decreased). The embryo mortality, at this concentration, was over 50% (Fogliano et al., 2022). The downregulation of apoptotic genes could be an indirect consequence of the inhibition of DNA repair mechanisms by DLZ, since the cells could not be stopped in G2, thus leading to an unmanageable situation. Another possible explanation comes from the dose-dependent increase in expression observed for *c-fos* and *c-jun*, which reach their maximum levels at the highest concentration. Both are members of AP-1 regulatory protein complex and once dysregulated, induce cell proliferation, differentiation, and malignant transformation. Their overexpression is typical of cells undergoing proliferation, and malignant differentiation (Chen et al., 2014; Priyadarshini et al., 2019). Moreover, among the proto-oncogenes, they are known to be early response genes (IEGs),

activated transiently and rapidly in response to a wide variety of environmental stimuli (Murphy et al., 2004). In light of these data, being in the embryogenetic phase, our results would indicate that at the higher DLZ dose, the embryos have scarce chance to repair the DNA and, therefore, maintain high levels of proapoptotic genes to eliminate the unrepairable cells and enough proliferative genes to substitute the death ones. Further information is necessary to understand what happens and an investigation clarifying the number and distribution of proliferating and apoptotic cells in the embryos would probably help.

The observed cell growth stimulation induced by DLZ, coupled with the inhibition of apoptosis and the deregulation of DNA repair genes expression could create a microenvironment for genomic instability which, ultimately, could lead to malignant transformation (Chen et al., 2014). *gadd, rad51 and mutL* abnormal expression was indeed related to unusual cell proliferation and observed in many tumor cells (Javid et al., 2018; Bonilla et al., 2020; Liu et al., 2015; Peng et al.,2015; Zhang et al., 2020). Overexpression of proto-oncogenes *c-jun* and *c-fos* has been found to be frequent in tumors (Chen et al., 2014; Priyadarshini et al., 2019). This hypothesis would fit perfectly with several animal studies reporting the inhibition of apoptosis and stimulation of tumor cell proliferation induced by benzodiazepines (Zhang et al., 2017). Moreover, the peripheral benzo receptors (PBR), with a pivotal role in the regulation of cell proliferation, are overexpressed in several types of tumors (Beinlich et al., 2000).

As postulated by Csoka & Szyf (2009), in addition to exerting effects by a purely pharmacological mechanism, many drugs also cause epigenetic changes that may or may not be beneficial. DNA methylation effects might be extremely teratogenic during embryogenesis, a critical period in which the DNA methylation pattern is highly susceptible to pharmaceutical interferences, as in the case of recognized teratogenic drugs strongly prohibited for pregnant women (van Gelder et al., 2010). The epigenetic changes introduced in the early stage of development might become noticed only later in life, transforming the adult health-related phenotype and, furthermore, abnormalities in DNA methylation caused by pharmaceutical drugs can produce defects in subsequent generations (Szyf, 2011; Sinclair et al. 2007).

Since most of the components of the methylation-mediated gene machinery are well conserved among *Xenopus* and humans (Meehan & Stancheva, 2001), these results ring an alarm bell regarding the long-term human use of benzodiazepines.

Tissue-specific investigations on head, gut and skeletal muscle are ongoing in order to ascertain and confirm the epigenetic effect more precisely. In the future, specific analyses will be carried out on the nervous system in order to highlight any abnormalities in the morphology and ultrastructure of the

brain, and different and more specific tests will be able to determine the behavioral consequences of an altered CNS.

Conclusion

In this study, a direct epigenetic activity exerted by the benzodiazepine delorazepam, a drug widely used and thus found as an environmental contaminant, was demonstrated. Alteration of the global methylation pattern is probably one the upstream cause of the mortality and teratogenesis found in *Xenopus laevis* embryos developed in contact with this drug, whose direct intervention on the promoters of key genes in embryonic development has been demonstrated, thus confirming the hypothesis of direct interference in the embryo development. The evidence of genomic instability with a probable induction to uncontrolled proliferation draws on the dose and the timing of administration that could give unexpected outcomes for human health but also emphasizes the importance of the assessment of the epigenetic effects of pharmaceuticals before their release in the commerce, and epigenotoxic effects of pollutants in the evaluation of water safety.

References

Anway, M. D., & Skinner, M. K. (2008). Epigenetic programming of the germ line: effects of endocrine disruptors on the development of transgenerational disease. Reproductive biomedicine online, 16(1), 23–25. https://doi.org/10.1016/s1472-6483(10)60553-6

Beby, F., & Lamonerie, T. (2013). The homeobox gene Otx2 in development and disease. Experimental eye research, 111, 9–16. https://doi.org/10.1016/j.exer.2013.03.007

Beinlich, A., Strohmeier, R., Kaufmann, M., & Kuhl, H. (2000). Relation of cell proliferation to expression of peripheral benzodiazepine receptors in human breast cancer cell lines. Biochemical pharmacology, 60(3), 397–402. https://doi.org/10.1016/s0006-2952(00)00325-7

Bilbul, M., Paparone, P., Kim, A. M., Mutalik, S., & Ernst, C. L. (2020). Psychopharmacology of COVID-19. Psychosomatics, 61(5), 411–427. https://doi.org/10.1016/j.psym.2020.05.006

Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H. M., Jiang, J., Marinelli, B., Pesatori, A. C., Bertazzi, P. A., & Yang, A. S. (2007). Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer research, 67(3), 876–880. https://doi.org/10.1158/0008-5472.CAN-06-2995

Bonilla, B., Hengel, S. R., Grundy, M. K., & Bernstein, K. A. (2020). RAD51 Gene Family Structure and Function. Annual review of genetics, 54, 25–46. https://doi.org/10.1146/annurev-genet-021920-092410

Boyadjieva, N., & Varadinova, M. (2012). Epigenetics of psychoactive drugs. The Journal of pharmacy and pharmacology, 64(10), 1349–1358. https://doi.org/10.1111/j.2042-7158.2012.01475.x

Burgio, E., Piscitelli, P., & Colao, A. (2018). Environmental Carcinogenesis and Transgenerational Transmission of Carcinogenic Risk: From Genetics to Epigenetics. International journal of environmental research and public health, 15(8), 1791. https://doi.org/10.3390/ijerph15081791

Chen, Y. Y., Zhu, J. Y., & Chan, K. M. (2014). Effects of cadmium on cell proliferation, apoptosis, and proto-oncogene expression in zebrafish liver cells. Aquatic toxicology (Amsterdam, Netherlands), 157, 196–206. https://doi.org/10.1016/j.aquatox.2014.10.018

Congras, A., Yerle-Bouissou, M., Pinton, A., Vignoles, F., Liaubet, L., Ferchaud, S., & Acloque, H. (2014). Sperm DNA methylation analysis in swine reveals conserved and species-specific methylation patterns and highlights an altered methylation at the GNAS locus in infertile boars. Biology of reproduction, 91(6), 137. https://doi.org/10.1095/biolreprod.114.119610

Csoka, A. B., & Szyf, M. (2009). Epigenetic side-effects of common pharmaceuticals: a potential new field in medicine and pharmacology. Medical hypotheses, 73(5), 770–780. https://doi.org/10.1016/j.mehy.2008.10.039

Cunha, D. L., Mendes, M. P., & Marques, M. (2019). Environmental risk assessment of psychoactive drugs in the aquatic environment. Environmental science and pollution research international, 26(1), 78–90. https://doi.org/10.1007/s11356-018-3556-z

Dee, C. T., Hirst, C. S., Shih, Y. H., Tripathi, V. B., Patient, R. K., & Scotting, P. J. (2008). Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. Developmental biology, 320(1), 289–301. https://doi.org/10.1016/j.ydbio.2008.05.542

Ding, N., Miller, S. A., Savant, S. S., & O'Hagan, H. M. (2019). JAK2 regulates mismatch repair protein-mediated epigenetic alterations in response to oxidative damage. Environmental and molecular mutagenesis, 60(4), 308–319. https://doi.org/10.1002/em.22269

Donkena, K. V., Young, C. Y., & Tindall, D. J. (2010). Oxidative stress and DNA methylation in prostate cancer. Obstetrics and gynecology international, 2010, 302051. https://doi.org/10.1155/2010/302051

Eden, A., Gaudet, F., Waghmare, A., & Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. Science (New York, N.Y.), 300(5618), 455. https://doi.org/10.1126/science.1083557

Fogliano, C., Motta, C. M., Venditti, P., Fasciolo, G., Napolitano, G., Avallone, B., & Carotenuto, R. (2022a). Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target Xenopus laevis. Aquatic toxicology (Amsterdam, Netherlands), 250, 106244. https://doi.org/10.1016/j.aquatox.2022.106244

Fogliano, C., Carotenuto, R., Panzuto, R., Spennato, V., De Bonis, S., Simoniello, P., Raggio, A., Avallone, B., Agnisola, C., & Motta, C. M. (2022b). Behavioral alterations and gills damage in Mytilus galloprovincialis exposed to an environmental concentration of delorazepam. Environmental toxicology and pharmacology, 97, 104030. Advance online publication. https://doi.org/10.1016/j.etap.2022.104030

Franco, R., Schoneveld, O., Georgakilas, A. G., & Panayiotidis, M. I. (2008). Oxidative stress, DNA methylation and carcinogenesis. Cancer letters, 266(1), 6–11. https://doi.org/10.1016/j.canlet.2008.02.026

Hernando, M. D., Heath, E., Petrovic, M., & Barceló, D. (2006). Trace-level determination of pharmaceutical residues by LC-MS/MS in natural and treated waters. A pilot-survey study. Analytical and bioanalytical chemistry, 385(6), 985–991. https://doi.org/10.1007/s00216-006-0394-5

Hoeijmakers J. H. (2001). Genome maintenance mechanisms for preventing cancer. Nature, 411(6835), 366–374. https://doi.org/10.1038/35077232

Hou, L., Zhang, X., Wang, D., & Baccarelli, A. (2012). Environmental chemical exposures and human epigenetics. International journal of epidemiology, 41(1), 79–105. https://doi.org/10.1093/ije/dyr154

Hu, J., & Yu, Y. (2019). Epigenetic response profiles into environmental epigenotoxicant screening and health risk assessment: A critical review. Chemosphere, 226, 259–272. https://doi.org/10.1016/j.chemosphere.2019.03.096

Hu, J., Liu, J., Li, J., Lv, X., Yu, L., Wu, K., & Yang, Y. (2021). Metal contamination, bioaccumulation, ROS generation, and epigenotoxicity influences on zebrafish exposed to river water polluted by mining activities. Journal of hazardous materials, 405, 124150. https://doi.org/10.1016/j.jhazmat.2020.124150

Javid, M., Sasanakietkul, T., Nicolson, N. G., Gibson, C. E., Callender, G. G., Korah, R., & Carling, T. (2018). DNA Mismatch Repair Deficiency Promotes Genomic Instability in a Subset of Papillary Thyroid Cancers. World journal of surgery, 42(2), 358–366. https://doi.org/10.1007/s00268-017-4299-6

Jones, P. A., & Gonzalgo, M. L. (1997). Altered DNA methylation and genome instability: a new pathway to cancer?. Proceedings of the National Academy of Sciences of the United States of America, 94(6), 2103–2105. https://doi.org/10.1073/pnas.94.6.2103

Kandi, V., & Vadakedath, S. (2015). Effect of DNA Methylation in Various Diseases and the Probable Protective Role of Nutrition: A Mini-Review. Cureus, 7(8), e309. https://doi.org/10.7759/cureus.309

Kellogg C. K. (1988). Benzodiazepines: influence on the developing brain. Progress in brain research, 73, 207–228. https://doi.org/10.1016/S0079-6123(08)60506-3

Lind, L., Penell, J., Luttropp, K., Nordfors, L., Syvänen, A. C., Axelsson, T., Salihovic, S., van Bavel, B., Fall, T., Ingelsson, E., & Lind, P. M. (2013). Global DNA hypermethylation is associated with high serum levels of persistent organic pollutants in an elderly population. Environment international, 59, 456–461. https://doi.org/10.1016/j.envint.2013.07.008

Lugoboni, F., Mirijello, A., Morbioli, L., Arzenton, E., Leone, R., Faccini, M., Casari, R., De Cosmo, S., Gasbarrini, A., & Addolorato, G. (2018). Does high-dose benzodiazepine abuse really produce liver toxicity? Results from a series of 201 benzodiazepine monoabusers. Expert opinion on drug safety, 17(5), 451–456. https://doi.org/10.1080/14740338.2018.1457646

Mattiuzzi, C., Sanchis-Gomar, F., & Lippi, G. (2022). Benzodiazepines consumption may have increased during the COVID-19 pandemic. Journal of affective disorders, 314, 124–125. https://doi.org/10.1016/j.jad.2022.07.017

McClure, E. A., North, C. M., Kaminski, N. E., & Goodman, J. I. (2011). Changes in DNA methylation and gene expression during 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced suppression of the lipopolysaccharide-stimulated IgM response in splenocytes. Toxicological sciences : an official journal of the Society of Toxicology, 120(2), 339–348. https://doi.org/10.1093/toxsci/kfq396

Meehan, R. R., & Stancheva, I. (2001). DNA methylation and control of gene expression in vertebrate development. Essays in biochemistry, 37, 59–70. https://doi.org/10.1042/bse0370059

Murphy, L. O., MacKeigan, J. P., & Blenis, J. (2004). A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration. Molecular and cellular biology, 24(1), 144–153. https://doi.org/10.1128/MCB.24.1.144-153.2004

Murphy, T. M., Mullins, N., Ryan, M., Foster, T., Kelly, C., McClelland, R., O'Grady, J., Corcoran, E., Brady, J., Reilly, M., Jeffers, A., Brown, K., Maher, A., Bannan, N., Casement, A., Lynch, D., Bolger, S., Buckley, A., Quinlivan, L., Daly, L., … Malone, K. M. (2013). Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients. Genes, brain, and behavior, 12(1), 125–132. https://doi.org/10.1111/j.1601-183X.2012.00865.x

Nunes, C. N., Dos Anjos, V. E., & Quináia, S. P. (2019). Are there pharmaceutical compounds in sediments or in water? Determination of the distribution coefficient of benzodiazepine drugs in aquatic environment. Environmental pollution (Barking, Essex : 1987), 251, 522–529. ttps://doi.org/10.1016/j.envpol.2019.05.015

Oligny L. L. (2001). Human molecular embryogenesis: an overview. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society, 4(4), 324–343. https://doi.org/10.1007/s10024001-0033-2

Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C. U., Jr, & Mohan, D. (2019). Pharmaceuticals of Emerging Concern in Aquatic Systems: Chemistry, Occurrence, Effects, and Removal Methods. Chemical reviews, 119(6), 3510–3673. https://doi.org/10.1021/acs.chemrev.8b00299

Pereira, A., Silva, L., Laranjeiro, C., Lino, C., & Pena, A. (2020). Selected Pharmaceuticals in Different Aquatic Compartments: Part I-Source, Fate and Occurrence. Molecules (Basel, Switzerland), 25(5), 1026. https://doi.org/10.3390/molecules25051026

Roth T. L. (2012). Epigenetics of neurobiology and behavior during development and adulthood. Developmental psychobiology, 54(6), 590–597. https://doi.org/10.1002/dev.20550

Santos, F., & Dean, W. (2004). Epigenetic reprogramming during early development in mammals. Reproduction (Cambridge, England), 127(6), 643–651. https://doi.org/10.1530/rep.1.00221

Sarangi A, Mcmahon T, Gude J (2021). Benzodiazepine Misuse: An Epidemic Within a Pandemic. Cureus 13(6): e15816. doi:10.7759/cureus.15816

Seo, M. S., Scarr, E., Lai, C. Y., & Dean, B. (2014). Potential molecular and cellular mechanism of psychotropic drugs. Clinical psychopharmacology and neuroscience: the official scientific journal of the Korean College of Neuropsychopharmacology, 12(2), 94–110. https://doi.org/10.9758/cpn.2014.12.2.94

Shah, K., Kamrai, D., Srinivas, S., Veluri, N., Chaudhari, G., Trivedi, C., Mansuri, Z. (2021). Benzodiazepine interaction with COVID-19 drugs. Prim Care Companion CNS Disord. 2021;23(6):21br03081. https://doi.org/10.4088/PCC.21br0308

Siegfried, Z., & Simon, I. (2010). DNA methylation and gene expression. Wiley interdisciplinary reviews. Systems biology and medicine, 2(3), 362–371. https://doi.org/10.1002/wsbm.64

Smith T. A. (2001). Type A gamma-aminobutyric acid (GABAA) receptor subunits and benzodiazepine binding: significance to clinical syndromes and their treatment. British journal of biomedical science, 58(2), 111–121.

Stancheva, I., El-Maarri, O., Walter, J., Niveleau, A., & Meehan, R. R. (2002). DNA methylation at promoter regions regulates the timing of gene activation in Xenopus laevis embryos. Developmental biology, 243(1), 155–165. https://doi.org/10.1006/dbio.2001.0560

Szyf M. (2011). The implications of DNA methylation for toxicology: toward toxicomethylomics, the toxicology of DNA methylation. Toxicological sciences : an official journal of the Society of Toxicology, 120(2), 235–255. https://doi.org/10.1093/toxsci/kfr024

Tapscott S. J. (2005). The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development (Cambridge, England), 132(12), 2685–2695. https://doi.org/10.1242/dev.01874

Toth M. (2021). Epigenetic Neuropharmacology: Drugs Affecting the Epigenome in the Brain. Annual review of pharmacology and toxicology, 61, 181–201. https://doi.org/10.1146/annurev-pharmtox-030220-022920

Tseng, H. T., Shah, R., & Jamrich, M. (2004). Function and regulation of FoxF1 during Xenopus gut development. Development (Cambridge, England), 131(15), 3637–3647. https://doi.org/10.1242/dev.01234

van Gelder, M. M., van Rooij, I. A., Miller, R. K., Zielhuis, G. A., de Jong-van den Berg, L. T., & Roeleveld, N. (2010). Teratogenic mechanisms of medical drugs. Human reproduction update, 16(4), 378–394. https://doi.org/10.1093/humupd/dmp052

van Vliet, J., Oates, N. A., & Whitelaw, E. (2007). Epigenetic mechanisms in the context of complex diseases. Cellular and molecular life sciences : CMLS, 64(12), 1531–1538. https://doi.org/10.1007/s00018-007-6526-z

Votaw, V. R., Geyer, R., Rieselbach, M. M., & McHugh, R. K. (2019). The epidemiology of benzodiazepine misuse: A systematic review. Drug and alcohol dependence, 200, 95–114. https://doi.org/10.1016/j.drugalcdep.2019.02.033

Wilkinson, J.L., Hooda, P.S.,Barker, J., Barton, S. & Swinden J. (2016) Ecotoxic pharmaceuticals, personal care products, and other emerging contaminants: A review of environmental, receptor-

mediated, developmental, and epigenetic toxicity with discussion of proposed toxicity to humans, Critical Reviews in Environmental Science and Technology, 46:4, 336-381, DOI: 10.1080/10643389.2015.1096876

Zhang, T., Yang, X., Zhou, J., Liu, P., Wang, H., Li, A., & Zhou, Y. (2017). Benzodiazepine drug use and cancer risk: a dose-response meta analysis of prospective cohort studies. Oncotarget, 8(60), 102381–102391. https://doi.org/10.18632/oncotarget.22057