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Chronic copper treatment prevents the liver critical balance transcription response induced by acetaminophen

Mauricio Latorre[a,b,c,d,*], Jason L. Burkhead[e], Christian Hodar[a,b], Miguel Arredondo[f], Mauricio González[a,b], Magdalena Araya[g,**]

[a] Laboratorio de Bioinformática y Expresión Génica, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile
[b] Center for Genome Regulation (Fondap 15090007), Universidad de Chile, Blanco Encalada 2085, Santiago, Chile
[c] Mathomics, Center for Mathematical Modeling, Universidad de Chile, Beauchef 851, 7th Floor, Santiago, Chile
[d] Instituto de Ciencias de la Ingeniería, Universidad de O'Higgins, Av. Viel 1497, Rancagua, Chile
[e] Department of Biological Sciences Anchorage, University of Alaska Anchorage, Anchorage, Alaska, United States
[f] Micronutrients Laboratory, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile
[g] Gastroenterología y Nutrición, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile

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ABSTRACT

The independent toxic effects of copper and acetaminophen are among the most studied topics in liver toxicity. Here, in an animal model of *Cebus capucinus* chronically exposed to high dietary copper, we assessed clinical and global transcriptional adaptations of the liver induced by a single high dose of acetaminophen. The experiment conditions were chosen to resemble a close to human real-life situation of exposure to both toxic stimuli. The clinical parameters and histological analyses indicated that chronic copper administration does not induce liver damage and may have a protective effect in acetaminophen challenge. Acetaminophen administration in previously non-exposed animals induced down-regulation of a complex network of gene regulators, highlighting the putative participation of the families of gene regulators HNF, FOX, PPAR and NRF controlling this process. This gene response was not observed in animals that previously received chronic oral copper, suggesting that this metal induces a transcriptional adaptation that may protect against acetaminophen toxicity, a classical adaptation response termed preconditioning of the liver.

1. Introduction

The human population is constantly exposed to environmental copper (water, food, copper utensils, among others), which is deposited mainly in the liver after regulated absorption. Nearly 20 years ago the World Health Organization (WHO) acknowledged the need to clarify the safe range of copper exposure for humans and its effects on human health. Metal content in the liver is considered the gold standard to determine global body copper status, a criterion also used for diagnosing some copper genetic disorders [1,2]. Considering the public health relevance and the difficulties of conducting these studies in humans, the first study in rhesus monkeys was performed in 2005 to define the effects of chronic copper exposure during early life, whereby the acute effects of copper exposure and those of sub-acute copper administration were initially assessed [3]. Under these conditions, no clinical evidence of copper toxicity was detected; however, at the fifth month of exposure, signs of cell damage were observed. Similarly, it was reported in 2012 that exposure of *Cebus capucinus* to high oral copper for three years did not impact their clinical or blood biochemical indicators, while liver histology remained unchanged as well, despite intense tissue copper deposits [4].

Whereas these studies on acute effects of copper greatly contributed to defining WHO safe copper content in water for human consumption [Copper in Drinking-water, World Health Organization, 2004), the WHO report maintained the statement that modifications of liver capacity to respond to chronic copper exposure and other stimuli remained unclear, and deserved further study.

The liver global adaptive response to different toxic conditions such as metals exposure, drugs effects, infections and/or genetic conditions has been an area of interest for several years and included various animal models [5–7]. Recently, it was demonstrated that treatment of rats with carbon tetrachloride (CCl_4, one of the most potent

* Corresponding author at: Gastroenterología y Nutrición, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile.
** Corresponding author.
E-mail addresses: mauricio.latorre@uoh.cl (M. Latorre), maraya@inta.uchile.cl (M. Araya).

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hepatotoxins) induces chronic liver damage, while a complex network of transcription factors is also activated, reprogramming the expression of these regulators in an adaptation process called 'critical balance of transcription factors of the liver' [8]. This phenomenon is characterized by gene regulation reprograming, where damaged cells return to normal function by re-expression of the families of transcription factors HNF, FOX and PPAR, involved in the regulation of liver genes, cell proliferation and basal metabolism respectively. This process is similar to that which occurs in somatic cells becoming pluripotent, supporting the idea that under damaging conditions, liver cells are able to reprogram global gene expression [9]. It is not known whether this particular liver transcriptional capacity is triggered by other drugs or toxic products, including metals present in human daily life.

Acetaminophen is frequently used to treat pain and fever. When ingested in high doses, it quickly induces a loss of hepatic function, and is one of the most common causes of acute liver failure (ALF) [10]. This response represents one of the most studied models of drug-induced liver injury [11]. It has also been demonstrated that a direct relationship exists between the toxicity induced by acetaminophen and oxidative stress damage in the liver. The administration of this drug induces increased levels of nitric oxide and reactive oxygen species, decreasing the concentration of intracellular reduced glutathione (GSH) level in liver, which have toxic effects that can also be inhibited by the biological antioxidant alpha-lipoic acid [12]. Knockdown of the superoxide dismutase 2 (SOD2, antioxidant enzyme) in rats exacerbates the toxic effect of acetaminophen (a single oral dose of 1000 mg/kg), increasing protein carbonyl and lipid peroxidation [13].

Taken together, these studies indicate that the ability of the liver to tolerate drug toxic effects depends on its capacity to respond to the oxidative stress damage. Since copper is a critical cofactor of superoxide dismutase and the oxidative stress response, but can also present a toxic challenge, our study aimed to test the hypothesis that a chronic copper exposure will precondition the liver response to a toxic drug challenge. We find that clinical and global transcriptional liver responses of *Capuchin* monkeys induced with a single high dose of oral acetaminophen are attenuated when the animals have been treated with chronic high dietary copper. This work indicates that the chronic challenge of copper pre-conditions the liver to limit injury.

2. Material and methods

2.1. Design and copper dosing

Animal handling and care was performed following the Principles for the Ethical Treatment of Non-Human Primates of the American Society of Primatologists and the recommendations of the United States Public Health Service *The Guide for the Care and Use of Laboratory Animals*, Eighth Edition. The Commission on Bioethics and Biosafety of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile and of the Institute of Nutrition and Food Technology (INTA), Universidad de Chile, approved the study protocol. All experimental procedures were performed in the facilities of the Chilean Primate Center, Pontificia Universidad Católica de Chile.

Eight healthy adult Cebus capucinus from the Chilean Primate Center, Pontificia Universidad Católica de Chile, were used in the study. The animals were 3–3.5 years of age at initiation of the study and were maintained indoors in the Primate Center, Pontificia Universidad Católica de Chile. Animals were in individual squeeze-back cages (height 77 cm; width 61 cm and depth 66 cm), housed under 14 h light, 10 h dark (LD 14:10, lights on at 0700 h) and constant environmental temperature and humidity (27 °C and 80%, respectively). In the room, the cages are in two parallel rows allowing frontal visual contact between the animals. Environmental enrichment was available in the cages (ropes, small tree branches and occasional toys). Nursery and veterinary staff supervised health, daily activities, and food and water consumption, offered at libitum. Diet consisted of fresh fruits, eggs, pellets and biscuits [798.2 kcal per day and 27.6 g of proteins per day] [14–16]. The amount of food offered was in excess of the estimated daily consumption. Meals were given at 1200 and 1800 h. Copper was administered to 4 experimental animals and the other 4 received placebo of similar appearance. Copper dosing was initially set at 5 mg Cu/kg/d and within two months progressively increased to 7.5 mg /kg/d, which avoided salivation and loose stools (main acute manifestation due to high copper ingestion). Copper was administered as copper gluconate hidden in a piece of fruit covered with preserve. Two especially trained persons visually supervised consumption. After three years, copper was stopped and one month later, all animals received 120 mg acetaminophen/kg as a unique oral dose, offered with the same strategy as used for copper (acute challenge protocol). Acetaminophen dosing was determined based on the lower doses reported inducing acute toxic manifestations in humans. After additional supervision for seven days animals were reassessed and blood and liver samples obtained.

2.2. Clinical assessments and procedures

During both the chronic and acute protocols, clinical assessments included visual evaluation of weight, activities and sleeping time, wellbeing, playing time and appetite through the day. Blood samples (3 mL) were collected from the saphenous vein, after Ketamine sedation (Ketaset, 10 mg/kg, Wyeth Ayerst), every second month during the first year, every third month during the second and third year and after the one-week acute acetaminophen challenge. After overnight food deprivation, samples were obtained between 08:30 and 09:00 h. Blood analyses, enzyme activity assays and copper concentrations were measured as previously described [16]. Briefly, mean corpuscular volume, hemoglobin and total white blood cells counts were obtained by automatic hematology analyses (CELL-DYN 1700 Abbott Diagnostics, Abbott Park, IL, USA), and free erythrocyte protoporphyrin concentrations with ZP Hematofluorometer model 206D (AVIV Biomedical Inc., Lakewood, NJ, USA). Enzyme activity for serum aspartate aminotransferase, alanine aminotransferase and gammaglutamyl transferase were determined by commercial kits (Química Clínica Aplicada SA, Amposta, Spain). Serum and liver copper concentrations were measured by atomic absorption spectrometry (model 2280; Perkin-Elmer, Norwalk, CT, USA).

Surgical liver biopsies were obtained every third month during the first year, every six months during the second and third year and after one week acute acetaminophen challenge, initially from the left lobule and subsequently towards the right lobule, avoiding scar tissues. One biopsy was snap frozen, maintained at -80 C° and analyzed for total copper concentrations as previously described [3]. A second piece was included in paraffin and serial 5-micron sections were stained with hematoxylin-eosin (general hepatic architecture and histology) and rhodamine (copper deposits), following routine techniques.

2.3. RNA isolation, cDNA labeling and microarray hybridization

Total RNA extraction from liver tissue was carried out using Trireagent (Ambion, Austin, TX, USA), following manufacturer recommendations (four animals per each conditions). Genomic DNA in the RNA samples were digested with RNase-free DNase I (Ambion, Austin, TX, USA). Concentration, 28S/18S ribosomal RNA (rRNA) ratio and RNA integrity number (RIN) of purified RNAs were measured using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) with the RNA 6000 Pico Chip kit (Agilent). After confirming purity of RNA samples, one microgram of total RNA was used to amplify antisense RNA (aRNA) using the MessageAmp II mRNA amplification kit (Ambion, Austin, TX, USA). Two-channel microarrays (Cy3-Cy5) were performed following previous published data [17]. Control (placebo) was used as common reference probe in all the hybridization, labeled with Cy3 dye. Experimental conditions (copper and/or acetaminophen) were labeled

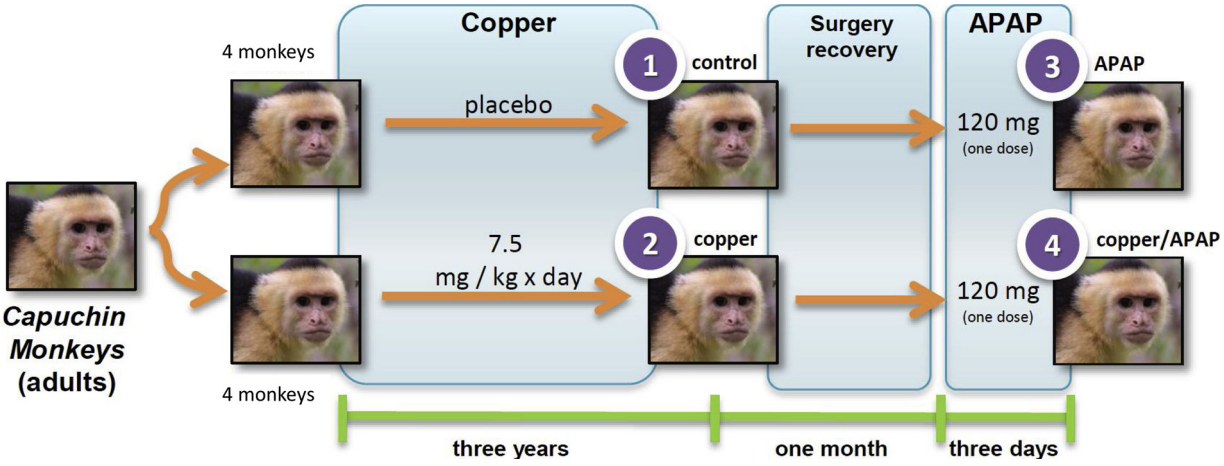

Fig. 1. Experimental design and clinical results. **A.** All the protocols were approved by the Institutional Animal Care Committee, Institute of Nutrition and Food Technology, University of Chile, which is based on the Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals (acetaminophen = APAP).

independently (four monkeys per each experiment) with Cy5 dye. Five micrograms aRNA from each sample was coupled with the dyes, according to manufacturer's instructions. Probe quantity and dye incorporation were assessed with a scanning spectrophotometer. Three different sets of hybridizations experiments were made (12 in total): a) aRNA from control (placebo condition) versus aRNA from copper (chronic loading); b) aRNA from control (placebo condition) versus aRNA from acetaminophen (acute-challenge); c) aRNA from control (placebo condition) versus aRNA from copper + acetaminophen (chronic loading and acute-challenge). For each experiment the two dye probes were mixed and concentrated to a volume of 50 ul in a solution, containing 20% formamide, 5x saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS); hybridization was performed according to microarray manufacturer's instructions. Slides were incubated for 16 h at 42 °C and a ScanArray GX laser reader (Perkin Elmer) detected fluorescent derivatives.

The overall expression of *Cebus capucinus* monkeys liver genes was performed using a Human MI Ready Array (catalog #HS1100, 49 K Human Genomic Array), from Microarrays Inc. (Huntsville, AL). Each array contained 48,958 70-mer oligonucleotides. Spot identification and quantification were performed with Scanarray Express software (Perkin Elmer). Array data were analyzed using the R statistical language and environment (http://www.r-project.org), specifically with the microarray analysis tools available by the Bioconductor Project (http://www.bioconductor.org). Data were background subtracted and normalized using the LIMMA Bioconductor package. Differentially expressed genes were determined using Significance Analysis of Microarrays (SAM) [18], with a false discovery rate (FDR) < 10% (S1 Table). We used GOrilla [19], a tool to identify enriched GO terms, to obtain biological attributes and over-represented gene expression information. All data is MIAME compliant and the genes described herein have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO number GSE93937. Validation of transcriptional changes were performed by quantitative real time PCR (qPCR) assay following previous experimental procedures [16].

2.4. Liver transcriptional regulatory network

The liver transcriptional regulatory network activated by copper and/or acetaminophen was generated by crossing-referencing information between the microarray data and a previous validated human global transcriptional gene network using a previous strategy protocol [20,21]. Briefly, the three experimental hybridizations were used as input data for Cytoscape software using iRegulon plug-in (Human network template, default parameters) [22]. Resulting transcriptional factor clusters were designed according to common enriched DNA binding site motif. Each cluster represents a family of regulators (S2 Table), which then were filtered according to reported validated experimental data [23]. All graph displays and network analyses were generated using the Cytoscape application [24]. Integration of the transcriptomic data into the metabolic model were done by iPath2.0 using enzyme classification (E.C) and KEGG: Kyoto Encyclopedia of Genes and Genomes identifiers from *Homo sapiens* genome assembly [25].

2.5. Statistical analysis

Statistical analyses were conducted by SYSTAT software 13.1. Differences were considered significant at $P < 0.05$. Immunohistochemical and histological data were analyzed by comparing serial liver sections from different times in a blind fashion.

3. Results

3.1. Chronic copper administration is protective in acetaminophen challenge

The experimental design included adult *Capuchin* monkeys fed daily 7.5 mg Cu/kg for three years (chronic copper protocol, details in material and methods) that seven days after finishing the chronic study received a unique dose of 120 mg acetaminophen/kg (acute protocol, Fig. 1). Animals were clinically supervised for one additional week at which time liver function, copper content and histology were assessed; acetaminophen dosing was decided on the basis of the lower ones reported inducing acute toxic manifestations in humans. This strategy allowed to assess four liver condition: i) control (placebo), ii) copper (chronic loading), iii) acetaminophen (acute-challenge) + placebo and iv) acetaminophen + copper (chronic loading and acute-challenge).

After all the protocols, *Capuchins* remained healthy, no changes in eating habits or physical activities were detected, not showing differences in term of body weight and food intake between monkeys fed with the control or the experimental diet. The analysis of hemoglobin, corpuscular volume and cell count were not affected after all the scenarios in study (Table 1). Serum aspartate aminotransferase (AST), alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT) activities were within the reported range for *Cebus paella* [26].

Serum aspartate aminotransferase (AST) activity was significantly increased at the end of the acetaminophen treatment compared to the control and copper only treatments (Table 1). The 2–3 fold increased AST is considered mild elevation, consistent with mild liver injury or

Table 1
Blood and tissue chemistry in adult monkeys in the assessed conditions.

	Control[†]	Copper[†]	Acetaminophen	Acetaminophen + Copper
Blood analysis				
Hemogoblin (g/L)	156 ± 1.3	147 ± 0.8	146 ± 1.1	140 ± 0.9
Mean corpuscular volume (%)	76.0 ± 0.2	75.8 ± 0.2	72.2 ± 0.3	74.8 ± 0.2
Total white cell count (x10E6)	8.8 ± 0.3	6.8 ± 0.2	7.50 ± 0.3	7.3 ± 0.4
Free erythrocyte protoporphyrin (μg/L RBC)	579 ± 16	757 ± 26	645 ± 21	698 ± 23
Liver enzymes (U/L)				
Serum aspartate aminotransferase	22.4 ± 2.6[a]	21.8 ± 2.4[a]	47.4 ± 6.8[b]	33.1 ± 4.1[a,b]
Alanine aminotransferase	24.5 ± 2.3	24.5 ± 2.0	28.8 ± 3.3	23.4 ± 3.2
Gammaglutamyl transferase	20.2 ± 1.9	24.5 ± 1.8	24.6 ± 2.9	29.5 ± 2.2
Copper concentration				
Serum copper (μmol/L)	846 ± 24.3	714 ± 24.0	801 ± 30.2	815 ± 23.5
Liver copper (nmol/g dry tissue)	217 ± 52.0	1160 ± 147[*]	203 ± 44.1	1105 ± 121[*]

[†] reference [8].
[*] t-test $p < 0.05$ ([a] vs acetaminophen, [b] vs control).

inflammation. The group treated with chronic copper exposure had a smaller increase in serum AST with acetaminophen challenge in relation to the control compared to the non-copper/acetaminophen group. This result is suggestive of a protective effect from chronic copper exposure.

Despite a significant increase in liver copper concentration, the three-year copper administration did not induce liver damage as indicated by clinical or biochemical analyses [27]. Liver histology as assessed by H&E stain was overall unremarkable, with normal cell size and no indication of necrosis or injury. Similar histology was observed at one week after the acute-challenge protocol with acetaminophen, with no visible induction of damage such as hepatocyte ballooning or necrosis, and no increased immune cell infiltration or structural change (Fig. 2). Rodanine staining indicated copper deposits in hepatocytes in copper-treated animals, with punctate staining in 25–50% of hepatocytes. Besides the serum AST, these results are consistent with the limited impacts of copper on clinical liver function indicators.

3.2. Global transcriptional response to acetaminophen is suppressed by chronic copper administration

The liver global transcriptional response to copper loading and acetaminophen challenge was determined through an integrative systems biology approach [20,28]. RNA microarray assays for each study condition were evaluated, comparing control (placebo) versus copper and/or acetaminophen data sets. A total of 17 genes changed its transcript abundance in response to the metal chronic loading, most encoding for basal metabolism and indicating a specific, limited transcriptional response to long-term copper administration.

Unlike the limited transcriptional response to chronic copper administration, a total of 352 genes were differentially expressed after the administration of a one-time dose of acetaminophen (120 mg/kg) to unexposed animals. The differentially expressed genes in this condition include over-representation of genes encoding for proteins involved in energy metabolism, gene regulation, signaling and cell proliferation, which indicates a multi-system transcriptional regulation response in

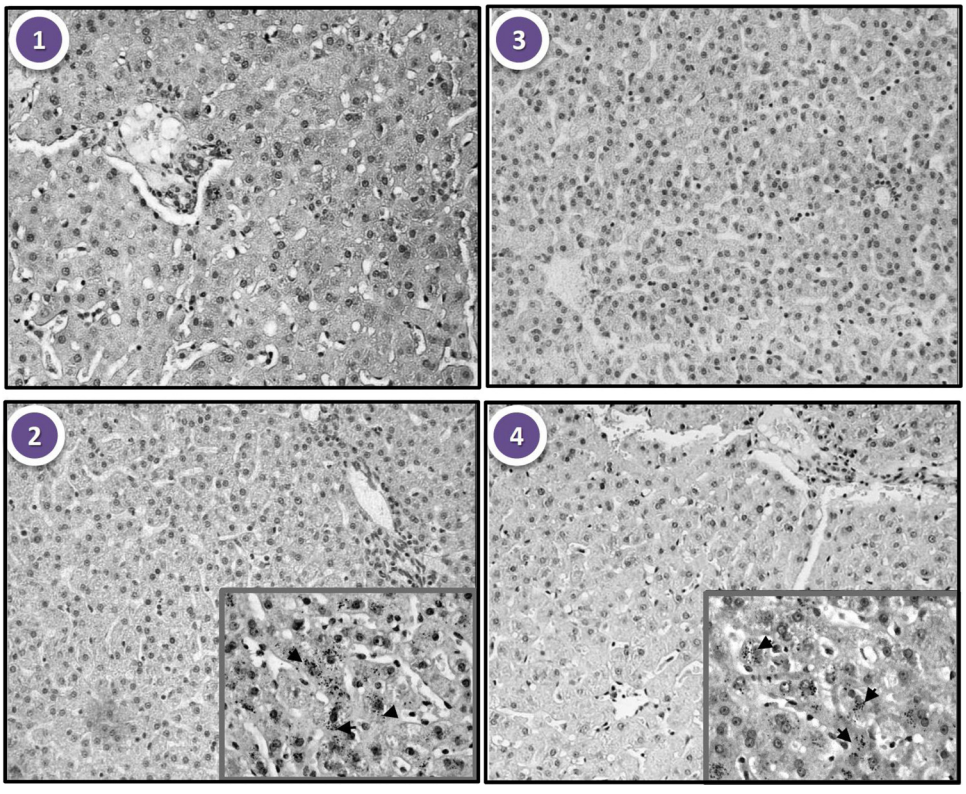

Fig. 2. Histological results. H&E staining (20X) indicates normal liver histology after three years receiving placebo (1), copper (2), placebo + single-dose acetaminophen (3) or copper + single-dose acetaminophen (4). Insets: arrow heads indicate copper deposits prior to and after acetaminophen challenge (Rhodanine staining 40X).

the liver. When acetaminophen was administrated to animals previously treated with copper, only a small number of genes increased expression (n = 16). This result indicates that the liver functional status after chronic copper exposure prevents the transcriptional response induced by acetaminophen. This phenotype is termed preconditioning of the liver, in which a prior event conditions the liver response to a second stressor [29].

3.3. An interconnected network links acetaminophen and copper transcriptomes through global regulators

Transcriptional regulatory networks have recently been developed as powerful tools to interpret global gene expression data, providing systems-level information about the logical configuration of the transcriptional response and identifying putative gene regulators that coordinate expression. This systems approach allows us to test the hypothesis that the gene expression responses to copper and acetaminophen administration are modulated by specific transcription factor families. Currently, a global human regulatory network model is available, which contains information about cellular identity (including liver) and function of complete and validated sets of transcriptional activators and corresponding gene targets [23].

Differential expression data from our copper/acetaminophen microarray study was integrated into the validated global gene regulatory network. The resulting model revealed a complex network of liver genes induced or repressed by copper and/or acetaminophen, connected to each other by common transcriptional activator families (Fig. 3).

The network covers more than 40% of the total differentially expressed genes (most of them representing the acetaminophen-only data). The general topological characteristic of the model indicated a classical in-degree power law distribution in the network structure, highly represented in other biological networks [30–33]. The chronic copper treated group minimally activated genes in the network, an expected result according to the microarray data. Acetaminophen administration to animals that received no previous copper, repressed a large set of genes in the network, most of them controlled mainly by global regulators of the TAF1 (basal transcriptional machinery), RFX5 (general immune response), SRF (cell development and proliferation) and nuclear factor families involved in general cell processes.

A gene ontology (GO) analysis indicates that processes involved in signaling pathways towards cell proliferation (MAPK/ERK) and gene expression control are over-represented in the network (Supp. Fig. S2).

Through the integration of the microarray data into a metabolic model, it was possible to identify putative affected metabolic pathways (activated or repressed) responsive to the copper and/or acetaminophen treatments (S3 Fig). In general, acetaminophen administration impacts the largest number of metabolic pathways, mainly down regulating genes involved in processes related to oxidative phosphorylation, lipid and carbohydrate metabolism.

Pentose phosphate metabolism is the primary pathway up-regulated by acetaminophen, and may be induced as a potential response to

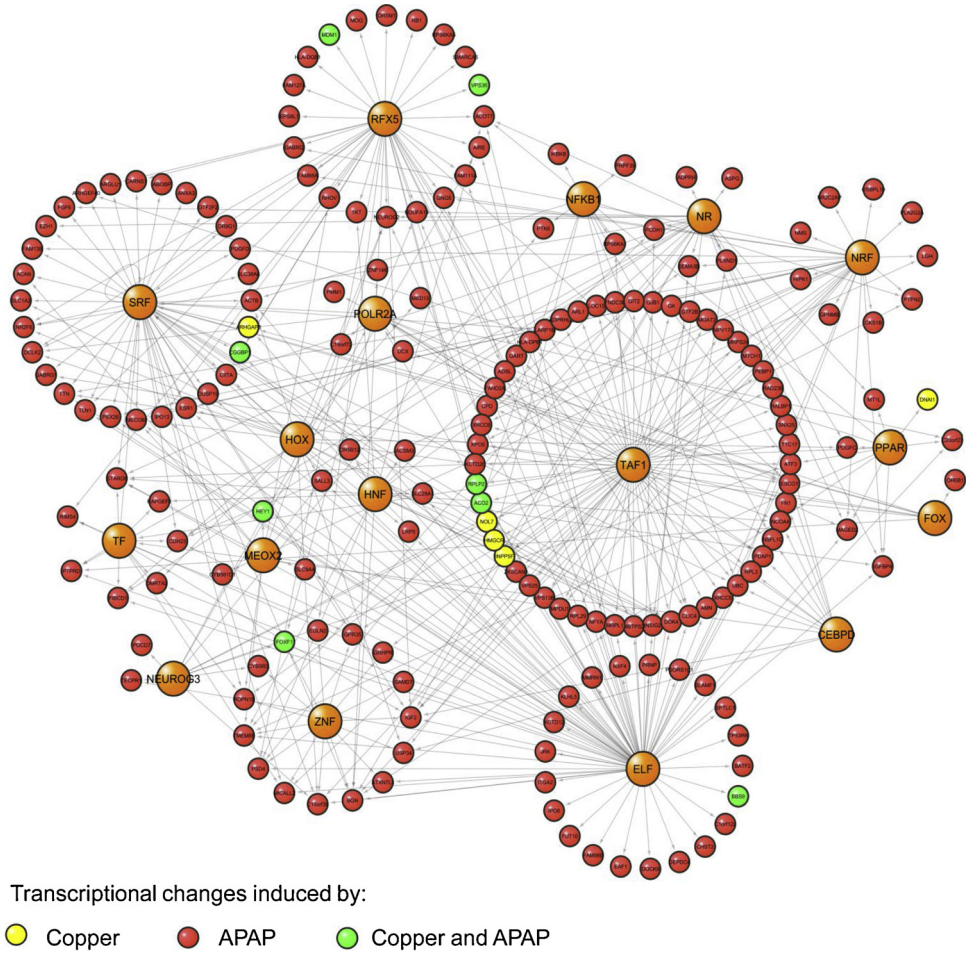

Fig. 3. Liver transcriptional regulatory network. The graphical model illustrates the complex network activated by copper and/or acetaminophen. The model contains 200 nodes connected (184 genes, 17 families of regulators) by 429 edges. Node colors represent the transcriptional changes (up or down regulated) during the copper and/or acetaminophen administration in relation to the placebo condition. **Orange** nodes indicate transcription factor families (names denote principal transcription factor families; specific details in supplementary information).

prevent oxidative stress damage through the metabolism of glutathione [34]. The oxidative stress transcriptional response was further evaluated by qPCR transcriptional changes of genes involved in oxidative stress response (S2 Fig). Both catalase (CAT) and thioredoxin (TRX) are up regulated during the acetaminophen administration, suggesting the presence of an oxidative liver stress and supporting the toxic effects of acetaminophen observed in other studies [35,36].

The metabolic model also highlights the participation of NRF and the ELF families of transcription factors that regulate the expression of lipid cofactors synthesis pathways (e.g. sphingolipid, SPTLC1) and hydrolysis of phospholipids (phospholipases, PLA2G2A). The transcriptional repression of these pathways supports the liver critical transcriptional balance model induced by acetaminophen and modulation by conditioning to copper exposure.

4. Discussion

The lack of change in liver enzymes in response to copper challenge suggests an adaptation to high levels of dietary copper and an overall lack of toxic effects. No previous studies have reported changes in copper homeostasis during the administration of acetaminophen. The combined treatment of copper and acetaminophen did not impact serum or liver copper content compared to the chronic loading of the metal. Liver histology and clinical liver function data indicates that chronic copper administration in the diet is well-tolerated by the liver and that the acetaminophen dose induces cellular damage indicated by AST, but not severe enough to cause necrosis of liver tissue.

Previous global gene expression studies addressed the transcriptional response of hepatoma cell cultures (HepG2) treated with high concentrations of copper [37,38]. The direct effect of the copper on the cells in culture was induction of a complex response, up- and down-regulating a high number of genes. The low number of activated genes after chronic (nontoxic) copper exposure in the *Capuchin* indicates that a transcriptional adaptation process occurred in the liver, likely in response to the potentially toxic effects of metal accumulation. The transcriptional adaptation phenotype was described previously in adult fishes that live in copper contaminated lakes, which do not show a significant liver transcriptional response after different copper treatments, suggesting the existence of an adaptive transcriptional response to chronic exposure to this metal [39].

A shorter-timeline preconditioning also was observed by O'Connor et al. [40], in the murine model. This study found that 24 h after the administration of an unique dose of the drug (600 mg/kg), liver from murine models induced changes in a total of 1788 genes. Animals preconditioned with a 400 mg/kg dose of acetaminophen prior to the 600 mg/kg challenge only induced expression changes in 156 genes after 24 h.

The data suggests an adaptive mechanism evolved to prevent liver damage in general, derived from different toxic inductors like metals or drugs [41]. Similarly, the studies indicate that a pre-conditioned liver can resist additional acute stress, even if the stressors are chemically distinct. Network analysis of genes differentially expressed in our treatment conditions revealed a critical balance transcriptional response in metabolic pathways induced by acetaminophen and a strikingly limited response when animals were pre-treated with copper. Most differentially expressed genes were down-regulated with acetaminophen challenge with signaling pathways towards cell proliferation (MAPK/ERK) and attenuation of gene expression are over-represented in the network (Fig. 3; Supp. Fig. S1). The down-regulation phenotype induced by acetaminophen is consistent with the proposed critical balance described in liver damaged by phenobarbital and CCl_4 [8]. The nuclear factors and nuclear receptors families HNF, FOX and PPAR, involved in hepatocyte development are the main regulators implicated in reversing liver failure in this system are also represented in our network controlling down-regulated genes [8]; supporting the hypothesis that acetaminophen induced a set of transcription factors previously identified with roles in modulating liver damage. In addition, nuclear factors (NRF) have been linked to a protective role during the adaptation process to the acetaminophen [40].

5. Conclusion

A three-year copper treatment in animals did not generate evidence of liver damage, while the copper treatment prevented a significant transcriptional response to acetaminophen challenge. We hypothesize that the liver adapted to chronic copper exposure and prevented down-regulation of genes involved in liver adaptation or regeneration. These in vivo responses are in contrast to results obtained in cellular models acutely exposed to toxic copper concentrations, where cells induce a large number of genes, mainly related with oxidative stress damage, copper homeostasis and cell protection [38]. Thus, the acetaminophen acute-challenge shows (for the first time) that liver capacity for transcriptional response is heavily influenced by a long-term preconditioning environment [29]. The *Capuchin* monkey model and all experiments specifically described here represent a situation similar that encountered in humans, enhancing the potential of this animal model to gain insight into human diseases and inform translational studies.

Competing interests

All the authors of this work declare to have no conflict of interest.

Authors' contributions

ML, JB and MA designed the research and wrote the paper; ML, CH, MG and MAr conducted the research and analyzed data; MA is responsible for the manuscript. All authors read and approved the final content.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.02.007.

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