Pharmacological Reports
2011, 63, 1435–1441
ISSN 1734-1140

Paracetamol treatment increases telomerase activity in rat embryonic liver cells

Augustinus Bader, Oliver Petters, Mario Keller, Sanja Pavlica

Biotechnological Biomedical Center, Department of Cell Techniques and Stem Cell Biology, University of Leipzig, Deutscher platz 5, 04103 Leipzig, Germany

Correspondence: Sanja Pavlica, e-mail: sanja.pavlica@bbz.uni-leipzig.de

Abstract:
Although paracetamol is known to have a damaging effect, this pharmaceutical is widely applied to pregnant and lactating women. Despite substantial progress in our understanding of its hepatotoxicity, some mechanisms, particularly of its embryonal and developmental toxicity, are still unknown. Thus, cell culture assays that investigate its toxicity are of particular interest. We assessed the effects of acute paracetamol treatment on cell viability (LDH assay, MTT assay), glutathione content (GSH assay), metabolic status (albumin and urea assays) and telomerase activity using rat embryonic liver cells (RLC-18 cells).
Incubation with low (6 mmol/l) and high (15 mmol/l) concentrations of toxin for 24 h leads to 20% and 50% cytotoxicity, respectively. Paracetamol exerted its toxicity in a similar pathway (depletion of GSH stores) as in adult liver cells, producing damage at the cellular level. Interestingly, paracetamol treatment significantly enhanced telomerase activity. Mechanisms involved in paracetamol-induced inhibition of cell senescence should be further elucidated. Telomerase activity in RLC-18 cells offers unique opportunities for examining basic biologic mechanisms. Our findings should encourage further studies to investigate a link between telomerase activity and toxicity, implying a role of impaired telomerase activity in human pathology.

Key words:
paracetamol, hepatotoxicity, telomerase activity, liver progenitors

Abbreviations: GSH – glutathione, MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, NAC – N-acetylcysteine, OS – oxidative stress, RLC-18 cells – rat embryonic liver (E-17) cells

Introduction

Excessive use of paracetamol (acetaminophen, APAP) can damage multiple organs, especially the liver and kidney. Its developmental toxicity is a medical concern. Moreover, paracetamol has been used routinely during all stages of pregnancy to reduce high temperatures and for pain relief. Since the early 1980s, the first reports on its overdose in pregnancy were published [2, 9]. Since paracetamol crosses the human placenta [8], the fetus is also at risk for toxicity from the parent compound, particularly after maternal overdose. Paracetamol overdose leads to the accumulation of N-acetyl-*p*-benzoquinoneimine (NAPQI), which undergoes conjugation with the natural antioxidant glutathione (GSH), leading to cell damage and death. Although oxidation by the fetal liver is ten-fold slower compared to adult liver, human fetal microsomes and isolated fetal hepatocytes are able to metabolize paracetamol into toxic NAPQI [14].

A detailed analysis of genes in the liver of rats of different ages after paracetamol administration showed that some of the age-related differences were due to

their time course and not their extent, i.e., the stress responses occurred earlier in young rats compared to the adults [10].

To date, large variations in toxicity are evident from many *in vitro* and *in vivo* studies in animals due to different paracetamol concentrations [7, 15, 17, 19]. Despite substantial progress in our understanding of its hepatotoxicity, some mechanisms, particularly of its developmental toxicity, still remain unknown.

Presently, using cells from fetal human liver is ethically problematic and great difficulties in obtaining such cells are expected. Rat embryonic (E17) liver (RLC-18) cells have been isolated and characterized by Takaoka and co-workers [18] who found that the activities of pyruvate kinase, glucose-6-phosphate dehydrogenase, hexokinase, gluco-kinase and catalase correspond to values typical of liver tissues of rat embryos in the later stage of pregnancy. Interestingly, the ability of a fetus to metabolize paracetamol after a maternal overdose begins at 18 weeks gestation and continues to increase through 23 weeks [9, 14]. Here, we used the RLC-18 cell line as a model of liver progenitor cells to examine the effects of paracetamol treatment during late pregnancy in rats. Different cellular assays were used to ascertain the effects of paracetamol on cell viability, metabolic status and telomerase activity.

Materials and Methods

Materials

Paracetamol, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), GSH, NAC, rat-albumin and collagenase were purchased from Sigma-Aldrich (Munich, Germany). RPMI Medium, penicillin, streptomycin and fetal calf serum (FCS) were obtained from Gibco Invitrogen (Karlsruhe, Germany), while accutase was from Biochrom AG (Berlin, Germany). All other chemicals were of analytical grade and obtained from reputable sources. Cell culture plates were manufactured by Becton Dickinson GmbH (BD, Germany).

Cell culture

The commercially available (ACC 332) rat embryonic liver (E-17) cell line (RLC-18) was utilized in this study. Cells were maintained in RPMI 1640 medium containing 5% FCS, penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. One day after seeding, the cells were treated with paracetamol for 24 h. Thus, all assays were conducted 48 h after cell seeding. The cells were cultured for 10–20 passages on dishes precoated with collagen I (BD Biosciences, Germany).

Cytotoxicity assays

Cytotoxicity was assessed by measuring the release of lactate dehydrogenase from the cells (LDH activity), based on our previous reports [12, 13].

The MTT assay was used because tetrazolium salts are cleaved to form a formazan dye only by metabolically active cells and are especially useful for assaying the quantification of viable cells. At the end of the incubation period, the medium was aspirated, and the cells were washed. MTT (0.5 g/l) was dissolved in medium and added to each well for the estimation of mitochondrial activity as described previously [11]. The optical density of each well was measured using an automatic microplate reader (Tecan, Switzerland) with a 630 nm reference wavelength and a 570 nm test wavelength. The results were expressed as the percentage of MTT reduction (cytotoxicity), assuming the absorbance of control wells as 0%.

Determination of total glutathione (GSH) levels

To check whether the toxic pathway in embryonic cells is similar to that in adult liver cells, we determined the total GSH content in cell homogenates using a GSH assay according to a previously reported method [11]. The GSH assay utilizes a carefully optimized enzymatic recycling method that uses GSH reductase for the quantification of GSH. The procedure was a minor modification of that previously employed in our laboratories. The absorbance measurements were performed at 412 nm by a Tecan microplate reader (Switzerland). Both reduced (GSH) and oxidized (GSSG) states of GSH were measured, and the assay reflects total GSH.

Determination of metabolic activity

The rate of albumin secretion was measured in medium supernatant of hepatocytes using our previously described enzyme-linked immunosorbent (ELISA)

assay [12, 13]. Urea concentration was assayed by the enzymatic urease method (Sigma-Aldrich, Munich, Germany).

Determination of telomerase activity

Telomerase activity was evaluated using the Telomerase ELISA Assay Kit TeloTAGGG (Roche Applied Science, Mannheim, Germany Cat. No. 11854666910). This photometric enzyme immunoassay utilizes the Telomeric Repeat Amplification Protocol (TRAP), as we previously reported [12]. The ELISA technique uses a biotinylated primer to immobilize the TRAP reaction products within a streptavidin-coated microplate and a specific DIG-labeled probe for detection. Following the addition of paracetamol to plated embryonic liver cells, cells were lysed and their telomerase activities were determined according to the manufacturer's instructions. The absorbance at 450 nm was determined. To confirm product specificity, a negative control was performed by heat inactivation of telomerase at 85°C for 10 min.

RT-PCR analysis of proto-oncogene markers

Two days after seeding, RNA was isolated from cells using the RNeasy-Kit (Qiagen). cDNA was transcribed from RNA with MLV transcriptase (Promega), random primers and dNTP mix. Real-time PCR was performed with 5 µg cDNA prepared from two independent isolations. TaqMan gene expression assays (fluorescently labeled, Applied Biosystems) were used with the following cDNA-specific primers and probes: c-fos (Rn02396759 m1), c-myc (Rn00561507 m1) and Bcl-2 (Rn99999125 m1). The TaqMan gene expression assay for eukaryotic 18S DNA was used as a reference (endogenous control).

Statistical analysis

The data are represented as the mean values SD of 3–6 independent experiments in each category. The results of the MTT, LDH, urea, albumin, GSH and telomerase assays were analyzed for variance using one-way ANOVA (SigmaStat software package, Jandel Corp., San Rafael, CA); $p < 0.05$ was considered to be statistically significant.

Results

The effect of paracetamol on cell viability

The high viability of rat embryonic liver cells maintained on collagen culture dishes is presented in Figure 1. Cultures of RLC-18 cells were treated with 5–30 mM paracetamol for 24 h in order to investigate sensitivity to paracetamol overdose. Interestingly, concentrations of paracetamol below 6 mmol/l and treatments of less than 24 h were not cytotoxic to RLC-18 cells (data not shown). Following the exposure to 6 and 15 mmol/l paracetamol for 24 h, cell viability (mitochondrial function) decreased to 80% and 50% of controls, respectively, as assessed by MTT assay (Fig. 1A). Thus, we chose these two concentrations of paracetamol, which caused mild (20%) and moderate (50%) cell death. Similarly, 24 h treatment with low (6 mmol/l) and high (15 mmol/l) doses of paracetamol induced slight to moderate morphological cell alteration, respectively, as evidenced by light microscopy (Fig. 2).

The rate of LDH release revealed a similar number of viable liver cells upon paracetamol treatment. Figure 1B shows a correlation between the increase in LDH activity and the toxin concentration.

In addition, viability of RLC-18 cells was indirectly measured by the GSH assay (Fig. 1C). We observed a drop in the intracellular GSH content with increasing paracetamol concentrations, indicating that the loss of GSH correlates well with cytotoxicity.

The effect of paracetamol on metabolic activity

The effect of paracetamol on liver-specific functions expressed in terms of urea synthesis and albumin production is presented in Figure 3. The addition of low doses of toxin showed a minor effect on the metabolic activity of RLC-18 cells.

Interestingly, the high concentration of paracetamol (15 mmol/l) had a greater effect on albumin (Fig. 3A) than urea values (Fig. 3B). Despite moderate toxicity after exposure to high dose of toxin, liver progenitors synthesized significantly higher amounts of urea in comparison with albumin production, showing that albumin levels correlated well with cell viability.

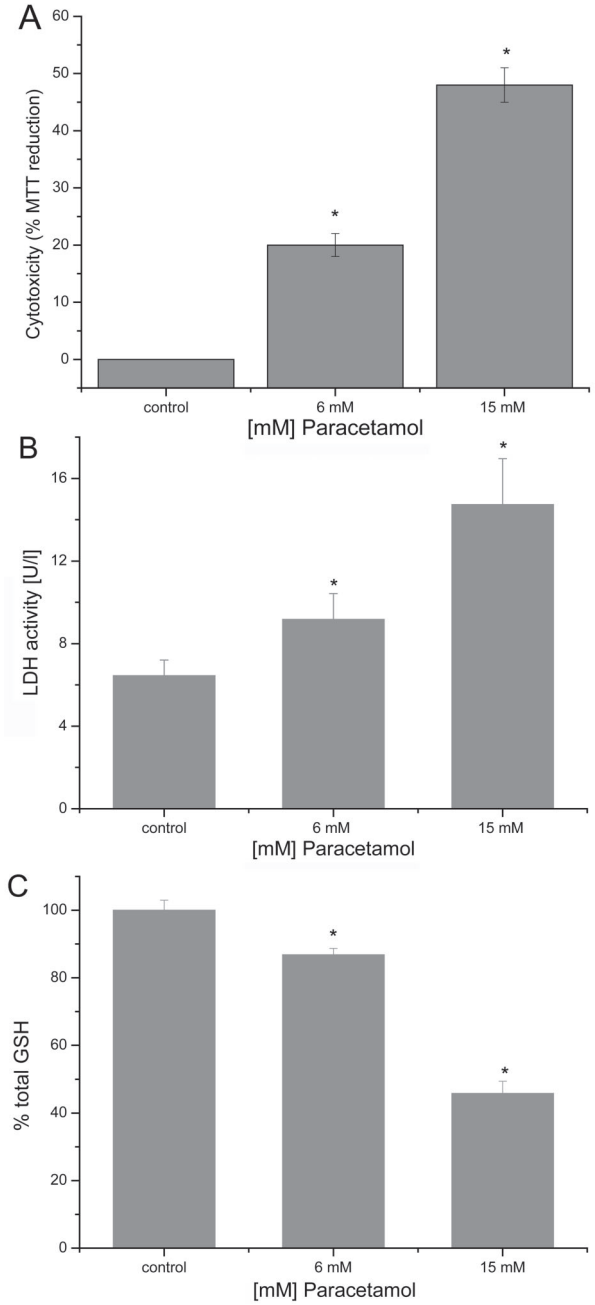

Fig. 1. Effect of paracetamol on RLC-18 cell viability as assessed by MTT reduction (**A**), lactate dehydrogenase (LDH) release (**B**) and glutathione (GSH) levels (**C**) in RLC-18 cells. The cells were seeded into collagen-coated plates and grown overnight. The following day, paracetamol (6 mmol/l and 15 mmol/l) was added for the next 24 h. In the MTT assay (**A**), cytotoxicity was designated as 0% in control wells without any treatment with paracetamol (cell viability was set to a maximum of 100%). In the GSH assay (**C**) the concentration of total GSH in untreated controls was set to 100%. The rate of LDH release (**B**) correlated well with the decrease in cell viability as indirectly measured by mitochondrial function (**A**). Bars represent the means ± SD of 4–6 independent experiments with evaluations performed in triplicate (* $p < 0.05$ *vs.* the untreated control, determined by one-way ANOVA)

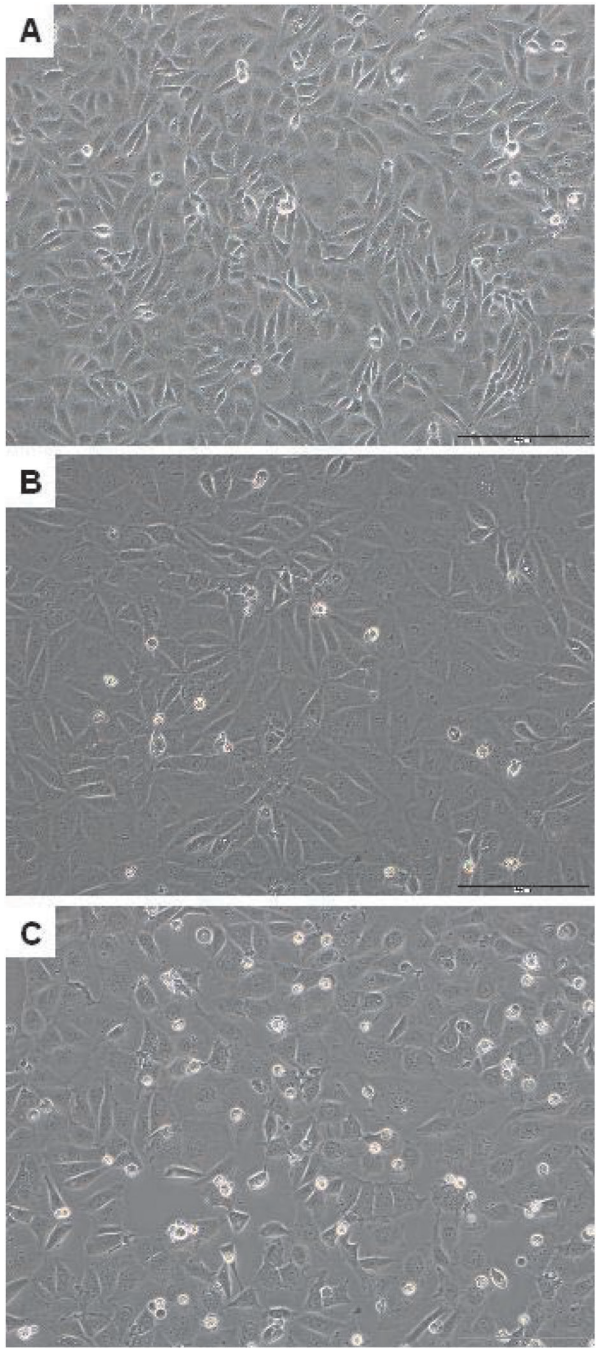

Fig. 2. Phase contrast images of RLC-18 cells cultured in collagen-coated plates without (**A**) and upon treatment with 6 mmol/l (**B**) and 15 mmol/l paracetamol (**C**). Representative pictures from four separate experiments are shown (original magnification × 100)

The effect of paracetamol on telomerase activity

Given that hepatocyte proliferation is a common feature of liver injury, this ability may require adequate telomere length and telomerase activity. Thus, we de-

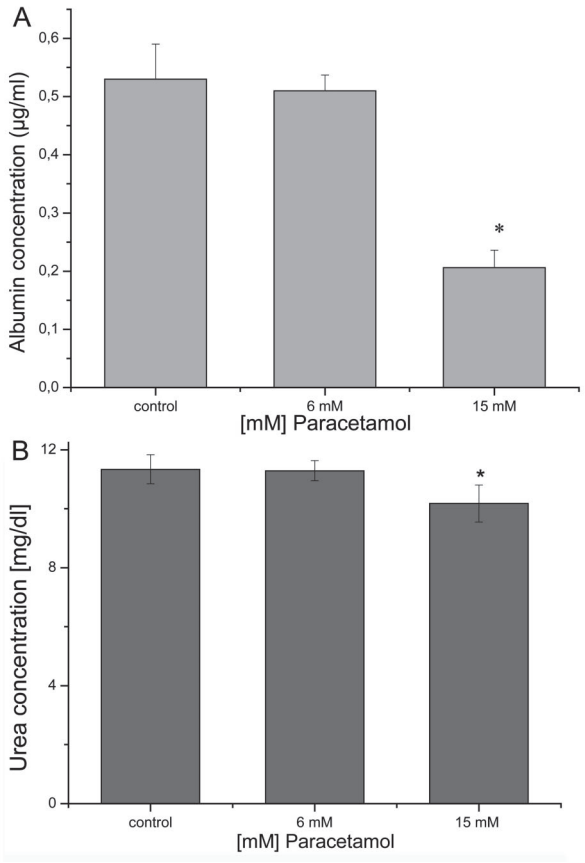

Fig. 3. The effect of paracetamol on the metabolic activity of RLC-18 cells. (**A**) Albumin production and (**B**) urea secretion were measured one day after the treatment of embryonic liver cells with 6 and 15 mmol/l paracetamol. The means of three independent experiments ± standard deviation are shown. One way analysis of variance (ANOVA) detected a statistically significant difference between the treatment groups (* $p < 0.05$ *vs.* untreated control)

termined whether telomerase activity is modulated by paracetamol overdose. Telomerase activity was detected in RLC-18 cells (at passages 10–20) and had similar values as the positive control (Fig. 4A). Our results unexpectedly showed that paracetamol treatment lead to strong telomerase activation. Paracetamol significantly altered telomerase activity of RLC-18 cells, even at a sub-toxic dose that caused slight cytotoxicity.

The effect of paracetamol on proto-oncogene markers

Acute hepatotoxicity induced by excessive doses of paracetamol may involve alteration of proto-oncogenes that regulate cell cycle progression or apoptosis. On the other hand, induction of telomerase activation might be a hallmark of cancer. Thus, we measured whether some proto-oncogenes are associated with the hepatotoxic response of liver progenitor cells to high doses of paracetamol used in this study. RT-PCR results (Fig. 4B) revealed the upregulation (RQ > 1) of c-fos, c-myc and bcl-2 genes (RQ = $2^{(-\Delta\Delta ct)}$) upon addition of paracetamol to cultured RLC-18 cells.

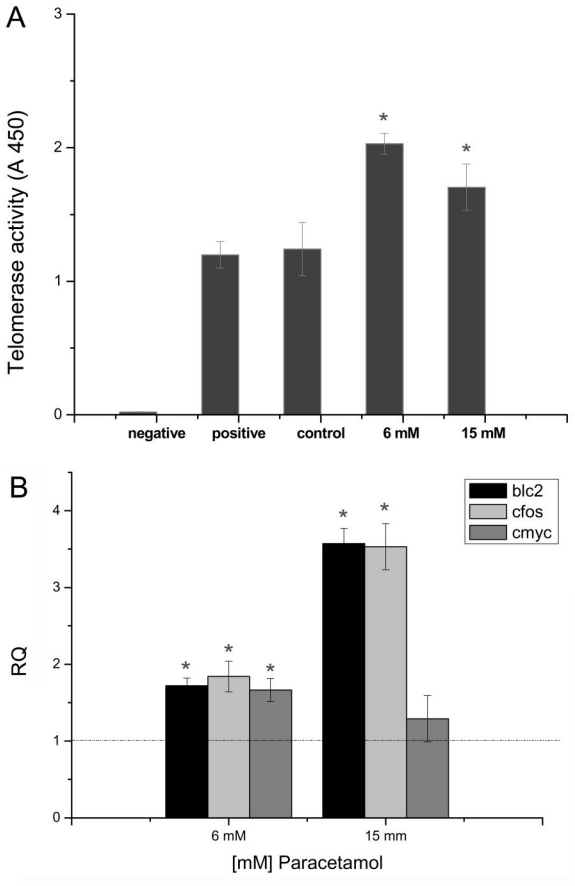

Fig. 4. (**A**) Effect of paracetamol treatment on telomerase activity of RLC-18 cells. Telomerase activity of embryonic liver cells measured one day after treatment with 6–15 mmol/l paracetamol. Unexpectedly, paracetamol significantly increased telomerase activity compared to the control. The values are the means of three separate experiments ± standard deviation (* $p < 0.05$ *vs.* untreated control, as revealed by the ANOVA test). (**B**) RT-PCR analysis of c-fos, c-myc and Bcl-2 gene expression in rat embryonic liver cells upon administration of paracetamol (6 and 15 mmol/l) for 24 h. RQ = $2^{(-\Delta\Delta ct)}$, RQ < 1 → downregulation; RQ > 1 → upregulation. Values in untreated controls are considered as 1. All other values are calculated relative to this. The mean ± SD (n = 2) are shown from experiments performed in triplicate. The paired Student's *t*-test was used to assess the significance of differences between observed data (* $p < 0.05$ *vs.* control)

Discussion

The effects of acute paracetamol treatment on cell proliferation (telomerase activity), GSH content, cell viability (MTT test, LDH activity) and ultrastructural alterations were investigated in a rat embryonic hepatic cell line (RLC-18 cells). Cells were exposed to low (6 mmol/l) and high (15 mmol/l) concentrations of paracetamol for 24 h. Under these conditions, cell death amounted to 20% and 50%, respectively, and slight to moderate morphological alterations were observed by light microscopy. Cytotoxicity, caused by 24 h exposure of RLC-18 to 15 mmol/l paracetamol, caused a 2-fold decrease in cell viability, intracellular GSH and albumin levels. Albumin production is one of the main functions of the liver. Most recently, we demonstrated that the synthesis of albumin in RLC-18 cells cultured on collagen was low compared to other liver embryonic and fetal cells [12]. These cells showed gene expression of albumin in a time-dependent manner, paralleling the hepatic differentiation process. Interestingly, the reduced capacity of albumin production of embryonic liver cells correlated well with the reduction of cell viability and GSH levels, especially at a concentration of 15 mmol/l paracetamol. Furthermore, our findings confirmed the role of GSH in cell survival. Thus, GSH reduction in paracetamol-treated progenitor cells could be explained, in part, as a consequence of mitochondrial damage, similarly as in adults [7].

To date, there has been no information about the difference in sensitivity towards paracetamol-induced cytotoxicity concerning embryonic to mature ages. Interestingly, we found that rat embryonic liver cells were more resistant to this toxin in comparison to primary rat hepatocyte cultures (data not shown). This finding is supported by the observation that other groups [15, 17] showed that mature rat hepatocytes responded to lower concentrations of paracetamol (0–10 mmol/l) in comparison with those used in the present study. Generally, the treatment line for paracetamol intervention is about 1 mM at 4 h post dose. Comparison of gene expression revealed that part of the protective response against paracetamol started earlier in young rats than in adults [10]. Rats are relatively immune to paracetamol since they convert most of the compound to the sulfate and the glucuronide metabolites [3]. This observed resistance might be partially due to the loss of CYP2E1, the primary metabolic enzyme for acetaminophen. Interestingly, we also found a pronounced downregulation of CYP2E1 gene levels in primary rat hepatocytes [13].

Mitochondria play an important role in cell death induced by many drugs, including hepatotoxicity from the overdose of paracetamol. BAX may be an early determinant of paracetamol-mediated hepatotoxicity, and BCL-2 overexpression unexpectedly enhances its hepatotoxicity [1]. The anti-apoptotic oncogene bcl-XL and the pro-apoptotic oncogene p53 are two key regulators of cell cycle progression and/or apoptosis, subsequent to DNA damage *in vitro* and *in vivo,* and can be altered by paracetamol. Further, paracetamol has been shown to increase c-jun and c-fos mRNA levels [6]. Thus, we tested the effect of paracetamol treatment on gene expression of proto-oncogenes in RLC-18 cells. Our findings showed that c-fos, c-myc and Bcl-2 gene expressions were upregulated, as assessed by RT-PCR. Upregulation of c-myc could lead to enhanced proliferation. Thereby, it is likely that the toxic effect of paracetamol on rat liver progenitors involves multiple mechanisms, including oxidative stress, upregulation of telomerases and alteration of pro-oncogenes.

Although the reason why telomerase is activated during the process of normal liver regeneration is still unclear, the telomere dysfunction is associated with defects in liver regeneration, and it accelerates the development of liver cirrhosis in response to chronic liver injury [16]. The upregulation of the telomerase activity in regenerating hepatocytes may, therefore, play an important role in the maintenance of liver functions. Therefore, in the context of liver regeneration, telomerase activation might be a cellular mechanism to confer an extended lifespan to replicating hepatocytes and hepatic progenitor cells [20]. On the other hand, high levels of telomerase activity are a hallmark of cancer [5], including hepatocellular carcinoma. Although telomerase activation seems to be associated with cancer progression, telomerase is not an oncogene, and controlled telomerase activation with a small molecule activator should not impose a cancer risk; this makes telomerase modulation an attractive approach for the treatment of chronic and degenerative diseases [4]. Under the conditions used in the present study, telomerase activation induced by paracetamol overdose may serve as a protective function by avoiding cellular senescence through the prevention of telomere shortening.

In conclusion, the obtained results demonstrated a moderate toxic effect of paracetamol on rat embryonic liver cells. We are currently investigating whether this model is sensitive enough to be used for evaluating toxicity of other hepatotoxins using a mainly proteomic approach. Despite the differences in species, our data imply an important role of impaired telomerase activity in human pathology. The inhibition of telomere shortening in RLC-18 cells by paracetamol overdose *in vitro* may improve the functional recovery of cells in a way that is important for potential cell therapy. Further investigation is needed to show the role of many other factors (stress resistance, pro-oncogenes, etc.) that have the potential to enhance telomerase activity and to connect it to developmental toxicity. Finally, future research should investigate the effects of other drugs on telomerase activity and cellular senescence.

Acknowledgments:
The work was funded by EU through the EU-project LIVEBIOMAT NMP3-CT-013653. Mrs. Angela Hennig (BBZ, University of Leipzig) is acknowledged for technical assistance. We are grateful to Prof. Dr. Michaela Schulz-Siegmund (Department of Pharmaceutical Technologies, University of Leipzig) for allowing us to perform RT-PCR at her lab.

References:

1. Adams ML, Pierce RH, Vail ME, White CC, Tonge RP, Kavanagh TJ, Fausto N et al.: Enhanced acetaminophen hepatotoxicity in transgenic mice overexpressing BCL-2. Mol Pharmacol, 2002, 60, 907–915.
2. Byer AJ, Traylor TR, Semmer JR: Acetaminophen overdose in the third trimester of pregnancy. JAMA, 1982, 247, 3114–3115.
3. Farkas D, Tannenbaum SR: Characterization of chemically induced hepatotoxicity in collagen sandwiches of rat hepatocytes. Toxicol Sci, 2005, 85, 927–934.
4. Harley CB: Telomerase therapeutics for degenerative diseases. Curr Mol Med, 2005, 205, 11.
5. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD: Specific association of human telomerase activity with immortal cells and cancer. Science, 1995, 268, 1115–1117.
6. Kitteringham NR, Powell H, Clement YN, Dodd CC, Tettey JN, Pirmohamed M, Smith DA et al.: Hepatocellular response to chemical stress in CD-1 mice: induction of early genes and γ-glutamylcysteine synthetase. Hepatology, 2000, 32, 321–333.
7. Larson AM: Acetaminophen hepatotoxicity. Clin Liver Dis, 2007, 11, 525–548.
8. Levy G, Garrettson LK, Soda DM: Evidence of placental transfer of acetaminophen. Pediatrics, 1975, 55, 895.
9. Ludmir J Main DM, Landon MB,Gabbe SG: Maternal acetaminophen overdose at 15 weeks of gestation. Obstet Gynecol, 1986, 67, 750–751.
10. Morishita K, Mizukawa Y, Kasahara T, Okuyama M, Takashima K, Toritsuka N, Miyagishima T et al.: Gene expression profile in liver of differing ages of rats after single oral administration of acetaminophen. J Toxicol Sci, 2006, 31, 491–507.
11. Pavlica S, Gebhardt R: Protective effects of ellagic and chlorogenic acids against oxidative stress in PC12 cells. Free Radic Res, 2005, 39, 1377–1390.
12. Pavlica S, Piscioneri A, Peinemann F, Keller M, Milosevic J, Staeudte A, Heilmann A et al.: Rat embryonic liver cell expansion and differentiation on NH_3 plasma-grafted PEEK-WC-PU membranes. Biomaterials, 2009, 30, 6514–6521.
13. Pavlica S, Schmitmeier S, Gloeckner P, Piscioneri A, Peinemann F, Krohn K, Siegmund-Schulz M et al.: Effect of native and NH_3 plasma-functionalized membranes on the gene expression profiles of primary hepatocytes. J Tissue Eng Regen Med, 2011, doi: 10.1002/term.453.
14. Rollins DE, von Bahr C, Glaumann H, Moldéus P, Rane A: Acetaminophen: potentially toxic metabolite formed by human fetal and adult liver microsomes and isolated fetal liver cells. Science, 1979, 205, 1414–1416.
15. Rousar T, Kucera O, Kriváková P, Lotková H, Kandár R, Muzáková V, Cervinková Z: Evaluation of oxidative status in acetaminophen treated rat hepatocytes in culture. Physiol Res, 2009, 58, 239–246.
16. Rudolph KL, Chang S, Millard M, Schreiber-Agus N, DePinho RA: Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. Science, 2000, 287, 1253–1258.
17. Shen C, Zhang G, Qiu H, Meng Q: Acetaminophen-induced hepatotoxicity of gel entrapped rat hepatocytes in hollow fibers. Chem Biol Interact, 2006, 162, 53–61.
18. Takaoka T, Yasumoto S, Katsuta H: A simple method for the cultivation of rat liver cells. Jpn J Exp Med, 1975, 45, 317–326.
19. Weeks BS, Gamache P, Klein NW, Hinson JA, Bruno M, Khairallah E: Acetaminophen toxicity to cultured rat embryos. Teratog Carcinog Mutagen, 1990, 10, 361–371.
20. Wege H, Brümmendorf TH: Telomerase activation in liver regeneration and hepatocarcinogenesis: Dr. Jekyll or Mr. Hyde? Curr Stem Cell Res Ther, 2007, 2, 31–38.

Received: March 23, 2011; **in the revised form:** June 28, 2011; **accepted:** July 1, 2011.