

## **The Effect of D-Galactosamine on Lean and Steatotic Rat Hepatocytes in Primary Culture**

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### **Short title:**

Effect of D-Galactosamine on Lean and Steatotic Hepatocytes

## **Summary**

The aim of our work was to compare the effect of D-galactosamine (GalN) on primary cultures of lean and steatotic rat hepatocytes isolated from intact and fatty liver, respectively. GalN caused more severe injury to steatotic hepatocytes than to lean cells as documented by lactate dehydrogenase leakage. Necrotic mode of cell death strongly prevails over apoptosis since we did not observe any significant increase in activities of caspase 3, 8 and 9 in any group of hepatocytes treated with GalN. Reactive oxygen species (ROS) formation and lipid peroxidation were elevated in a dose-dependent manner by GalN and were significantly more pronounced in fatty hepatocytes. A decrease in the percentage of hepatocytes with energized mitochondria was observed from 30 mM and 10 mM GalN in lean and steatotic hepatocytes, respectively. Our results undoubtedly indicate that steatotic hepatocytes exert higher sensitivity to the toxic effect of GalN. This sensitivity may be caused by more intensive GalN-induced ROS production and lipid peroxidation and by higher susceptibility of mitochondria to loss of mitochondrial membrane potential in steatotic hepatocytes. In our experimental arrangement, apoptosis does not seem to participate considerably on hepatotoxic action of GalN in either group of hepatocytes.

## **Key words**

Fatty liver • D-galactosamine • Hepatocytes *in vitro* • Hepatotoxicity • Oxidative stress

## Introduction

Non-alcoholic fatty liver disease (NAFLD) belongs to the broad group of obesity-related disorders. It is characterized by the accumulation of lipid droplets in the liver which arises as a consequence of insulin resistance. NAFLD is believed to be the hepatic component of metabolic syndrome and it is the most common chronic liver disease in the Western world (Vernon *et al.* 2011). NAFLD is histologically defined by the presence of liver steatosis exceeding 5% of hepatocytes (Kleiner *et al.* 2005). In addition to simple steatosis, NAFLD includes non-alcoholic steatohepatitis (NASH) which can progress to fibrosis and finally to end-stage liver disease.

Recently, some authors found that NAFLD is associated with higher risk of drug liver injury when compared to general population (Nguyen *et al.* 2008, Tarantino *et al.* 2007, Tarantino *et al.* 2009). This may be caused by polypragmasia in patients with obesity, metabolic syndrome and NAFLD, and/or by liver alterations induced by NAFLD predisposing the liver to toxic injury (Fromenty 2013). These changes include altered expressions and activities of biotransformation enzymes (i.e. cytochrome P450 2E1) (Merrell and Cherrington 2011), mitochondrial dysfunction (Kučera *et al.* 2011a, Perez-Carreras *et al.* 2003), reduced amount of hepatic glutathione content, increased oxidative stress and lipid peroxidation (Garcia-Ruiz *et al.* 2006, Kučera *et al.* 2011a, Videla *et al.* 2004), augmented susceptibility of hepatocytes to apoptosis and necrosis (Bechmann *et al.* 2010, Feldstein *et al.* 2003) and cytokine/adipokine dysbalance (Tilg 2010). All mentioned factors contribute also significantly to the pathogenesis and/or progression of NAFLD.

In our previous experiments, we have proven that rat fatty hepatocytes show greater susceptibility to the toxic effect of acetaminophen (Kucera *et al.* 2012b) and thioacetamide (Kučera *et al.* 2011b) *in vivo*, and acetaminophen (Kucera *et al.* 2012a) and tert-butyl

hydroperoxide (Garnol *et al.* 2014, Kučera *et al.* 2014) *in vitro*. Tert-butylhydroperoxide is a short-acting toxin causing relatively rapid damage by direct induction of oxidative stress in hepatocytes while acetaminophen for its toxic effect requires metabolic activation to reactive intermediate N-acetyl-p-benzoquinone imine. In comparison with tert-butyl hydroperoxide and acetaminophen, D-galactosamine (GalN) is a highly selective hepatotoxin with different mechanism of toxic action. GalN **primary** depletes the pool of uridine nucleotides in hepatocytes which inhibits transcription and protein synthesis in the liver (Keppler *et al.* 1970). This inhibition is **consequently** accompanied by increased oxidative and nitrosative stress, depletion of reduced glutathione and mitochondrial dysfunction (McMillan and Jollow 1992, Quintero *et al.* 2002, Ranchal *et al.* 2006). *In vivo*, GalN induces activation of innate immune response (i.e. Kupffer cells) by endotoxin (Stachlewitz *et al.* 1999) and strongly increases sensitivity of the liver to tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) (Lehmann *et al.* 1987). GalN induces both necrosis and apoptosis in the liver (Cuesta *et al.* 2006, Kuhla *et al.* 2009) and in hepatocytes in primary culture (Siendones *et al.* 2005).

Considering different mechanism of GalN toxic action, the aim of our study was to find out whether steatotic rat hepatocytes in primary culture exert also higher susceptibility to GalN than lean hepatocytes, similarly as in the case of acetaminophen and tert-butylhydroperoxide. The emphasis of this work was given on a role of oxidative stress and a mode of cell death in GalN toxicity.

## Materials and Methods

### *Chemicals*

William's E medium (without phenol red), foetal bovine serum, penicillin, streptomycin and glutamine were purchased from Biochrom Ltd. (UK). Collagenase (Collagenase NB 4 Standard Grade from *Clostridium histolyticum*) was obtained from SERVA Electrophoresis GmbH (Germany), insulin (Actrapid, Hoechst, Germany) and glucagon (Novo Nordisk, Denmark) were from the suppliers mentioned in brackets. D-galactosamine, type I collagen, trypan blue, dexamethasone and all other chemicals were, unless otherwise stated, of at least analytical grade and obtained from Sigma-Aldrich (WI, USA).

### *Animals*

Male albino Wistar rats (Velaz, Czech Republic) were housed at  $23 \pm 1^\circ\text{C}$ ,  $55 \pm 10\%$  relative humidity, air exchange 12–14 times/h, and 12-h light–dark cycle periods (6:00 h to 18:00 h). Rats used for isolation of non-steatotic hepatocytes were fed *ad libitum* a standard pelleted diet (Velas, Czech Republic; 10% energy fat, 30% energy proteins, 60% energy saccharides) for 6 weeks. For isolation of fatty hepatocytes, animals were fed a high-fat diet with 71% of energy derived from fat (lard), 18% from proteins and 11% from saccharides for 6 weeks. The animals had free access to tap water. All animals received care according to the guidelines set by the Animal-Welfare Body of the Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic, and the European Guidelines on Laboratory Animal Care. All animal experiments were approved by the committee mentioned above and by the Ministry of Education, Youth, and Sports of Czech Republic (authorisation reference number 1315-28572/2012-30).

### *Hepatocyte isolation, cultivation and treatment*

Hepatocytes were isolated from rat livers by two-step collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes used in experiments was more than

90% as confirmed by trypan blue exclusion test. Isolated hepatocytes were suspended in William's E medium supplemented with foetal bovine serum (6%), glutamine, antibiotics and hormones (Kucera *et al.* 2012a) and plated in collagen-coated 12-well plates ( $4 \times 10^5$  cells in 400  $\mu$ l/well, NUNC) and 96-well plates ( $3 \times 10^4$  cells in 100  $\mu$ l/well, NUNC).

Hepatocytes were allowed to establish monolayer in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> at 37 °C for 2h. Then the medium was replaced with a fresh supplemented medium without foetal bovine serum containing dissolved GalN (0 - 40 mmol/l). Based on our previous results, we have chosen treatment period of 24 hours. Then the medium was collected and cells were harvested for the required assays.

### *Biochemical assays*

Hepatocyte injury was determined by lactate dehydrogenase (LDH) leakage. LDH activity in the culture medium and in cell lysate (Cell Lysis Buffer, Cell Signalling Technology, MA, USA) was measured using a commercial kit from DiaSys (Germany). The production of reactive oxygen species (ROS) was evaluated using 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes, USA) as previously described (Kučera *et al.* 2014). Secondary end product of lipoperoxidation malondialdehyde (MDA) in culture medium was determined by the assessment of thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.* 1979). For better comparison of results from different experiments, values of ROS production and MDA concentration are expressed as percent of control non-steatotic hepatocytes (100 %).

Concentration of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in culture medium was measured by enzyme linked immunosorbent assay (ELISA) according to manufacturer's instructions (BMS622, Bender MedSystems, Austria). Results are expressed as percent of control non-steatotic hepatocytes (100 %).

The activities of caspases 3, 8 and 9 were assessed in cell lysate (Cell Lysis Buffer) after addition of specific substrates Ac-DEVD-AMC, Ac-LETD-AFC and Ac-LEHD-AMC, respectively (Enzo Life Sciences, NY, USA). The activities were measured in a fluorescent mode using a TECAN Infinite M200 spectrofluorometer (Tecan Group AG, Switzerland). The excitation and emission wavelengths were 350 and 460 nm for caspase 3 and caspase 9, and 400 and 505 nm for caspase 8. Results were normalized to protein concentration (Bradford 1976) and expressed as percent of control non-steatotic hepatocytes (100 %).

#### *Fluorescent microscopy*

Mitochondrial membrane potential (MMP) was depicted by fluorescence microscopy using JC-1 (Molecular Probes, Inc., Oregon, USA) as previously described (Kučera *et al.* 2014). Results are expressed as percentage of cells with energized mitochondria from all attached cells.

#### *Statistical analysis*

Results are expressed as means $\pm$ SD of at least three independent experiments;  $p < 0.05$  was set as the border for statistical significance. Statistical evaluation was performed using GraphPad Prism software 6.01 (La Jolla, CA, USA). Data were first tested for normality by means of D'Agostino & Pearson omnibus test. All data did not follow Gaussian distribution, thus the data were analysed by a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test.

## **Results**

Figure 1A shows LDH leakage from lean and steatotic hepatocytes after 24-hour incubation with GalN. In non-fatty cells, a significant increase in LDH leakage was observed

from 30 mM concentration of GalN ( $p<0.001$ ). In steatotic hepatocytes, LDH leakage was elevated from 20 mM concentration of GalN ( $p<0.05$ ). We did not observe any increase in caspase 3, 8 and 9 activities in cell lysate in any group treated with GalN when compared with an appropriate control (Figure 1B, 1C and 1D).

ROS production was elevated by incubation with GalN in a dose dependent manner in both lean and steatotic hepatocytes (Figure 2A). A significant increase in generation of ROS was found from 10 and 5 mM concentrations of GalN in non-steatotic ( $p<0.05$ ) and fatty cells ( $p<0.001$ ), respectively. In steatotic hepatocytes, ROS production was significantly higher than in lean cells (5, 10 and 20 mM GalN). MDA concentration in cell lysate is depicted in Figure 2B. Production of TBARS is dose-dependent. GalN at concentrations from 30 mmol/l induced a significant elevation of TBARS in steatotic hepatocytes, whereas only the highest tested dose of GalN (40 mmol/l) caused a significant MDA increase in non-fatty cells.

Figure 3 depicts concentration of TNF $\alpha$  in culture medium after 24 hours. Cultivation of hepatocytes with GalN led to a dose dependent increase in TNF $\alpha$  levels. Significant elevations of TNF $\alpha$  levels were observed similarly from 20 mM GalN in both lean and fatty hepatocytes.

In lean hepatocytes, GalN from concentration of 30 mmol/l ( $p<0.05$ ) induced a significant decrease in percentage of cells containing energized mitochondria (Figure 4). In contrast to this finding, steatotic hepatocytes exerted a significant reduction in the percentage from 10 mM GalN ( $p<0.05$ ). GalN at concentrations from 10 to 40 mmol/l induced more severe decrease in the percentage of hepatocytes with energized mitochondria in fatty cells. Figure 5 shows representative fluorescent microphotographs of lean and steatotic hepatocytes incubated without GalN or with 20 mM GalN.

## **Discussion**



Recently, several clinical studies showed that patients with NAFLD exert higher risk of drug-induced liver injury than general population (Nguyen *et al.* 2008, Tarantino *et al.* 2007, Tarantino *et al.* 2009). Also some experimental works confirmed higher susceptibility of fatty hepatocytes to toxic injury *in vivo* (Aubert *et al.* 2012, Kucera *et al.* 2012b, Kučera *et al.* 2011b, Sawant *et al.* 2006) and *in vitro* (Kucera *et al.* 2012a, Kučera *et al.* 2014, Luo *et al.* 2012). In the present study, we demonstrate that steatotic hepatocytes in primary cultures are more sensitive to the toxic effect of GalN.

D-galactosamine blocks transcription and protein synthesis in the liver via depletion of uridine nucleotides in hepatocytes (Keppler *et al.* 1970). Both modes of cell death, apoptosis and necrosis, participate on hepatotoxic effect of GalN *in vivo* (Cuesta *et al.* 2006, Kuhla *et al.* 2009). Transcriptional arrest of hepatocytes by GalN sensitizes liver to TNF $\alpha$  which is considered to be one of the main causes of GalN-induced liver injury (Leist *et al.* 1994). *In vivo*, sensitization of liver parenchymal cells to TNF $\alpha$  leads to the extensive apoptosis (Leist *et al.* 1994, Saavedra *et al.* 1997). Apoptosis was proved to be involved in GalN-induced rat hepatocyte damage *in vitro* as well (Siendones *et al.* 2005). Results of this manuscript show that necrosis strongly prevails over apoptosis in our experimental conditions. Intracellular activities of caspase 8 and caspase 9, the initiator caspases of the extrinsic and mitochondrial apoptotic pathways, respectively, showed only a non-significant trend of increased activities in the lowest tested dose of GalN (5 mmol/l) in lean and steatotic hepatocytes. Activity of caspase 3 in cell lysate was not increased in any group incubated with GalN. Our results of caspase 8 and 9 activities correspond to the results of Siendones (Siendones *et al.* 2005) who found that these caspases are not activated in GalN toxicity in rat hepatocytes *in vitro*. As concerns the activity of caspase 3, we did not observe any activation of this caspase in GalN-induced injury to rat hepatocytes which is in contrast to findings of Siendones (Siendones *et al.* 2005). Different results of caspase-3 activation by GalN may be explained by distinct

cultivation protocols. Whereas we exposed hepatocytes to GalN immediately after the seeding process, the other group of authors delayed administration of GalN to hepatocytes by 24 hours (Siendones *et al.* 2005). This surmise is supported by findings of Canova (Canova *et al.* 2008) who found that GalN is more potent to induce hepatocyte apoptosis during 24 to 48 h incubation than during 0 to 24 h period.

Necrotic death of hepatocytes was monitored by LDH leakage which was significantly increased from 30 mM and 20 mM concentration of GalN in non-steatotic and fatty hepatocytes, respectively. Significantly increased activity of caspase 8 in control fatty hepatocytes when compared to control lean cells together with mentionable trend of elevated activities of caspase 3 and caspase 9 in the same experimental group may indicate that steatotic hepatocytes predispose to apoptosis.

Although the role of TNF $\alpha$  in GalN toxicity *in vivo* is well known, the importance of TNF $\alpha$  in GalN-induced hepatocyte injury *in vitro* is not well understood. We did not find any difference in levels of TNF $\alpha$  in culture medium between lean and steatotic control hepatocytes which correlates well with the absence of significant differences in both serum (Kučera *et al.* 2011b) and hepatic (Kucera *et al.* 2012b) contents of TNF $\alpha$  between lean rats and animals with simple steatosis *in vivo*. Exposure of hepatocytes to GalN led to a similar dose-dependent elevation in TNF $\alpha$  concentration in both lean and fatty groups. Despite the significance of TNF $\alpha$  in apoptosis, we did not find noticeable activation of apoptotic mode of cell death in GalN-treated hepatocytes as mentioned above.

Oxidative stress and nitrosative stress were reported to contribute significantly to GalN toxicity (McMillan and Jollow 1992, Quintero *et al.* 2002, Ranchal *et al.* 2006). Oxidative stress does not seem to be caused primarily by GalN, but it is rather a consequence of metabolic disturbances induced by depletion of uridine nucleotides, inhibition of

proteosynthesis and synthesis of glutathione (McMillan and Jollow 1992, Quintero *et al.* 2002). Our study demonstrates that steatotic hepatocytes are more sensitive to the induction of generation of ROS and lipid peroxidation by GalN. All tested concentrations of GalN increased production of ROS in fatty cells whereas the lowest dose of GalN did not elevate significantly ROS formation in lean hepatocytes. Moreover, GalN (5 – 20 mmol/l) caused significantly higher generation of ROS in steatotic hepatocytes than in lean cells. We have previously demonstrated that steatotic hepatocytes *in vitro* exert higher production of ROS, increased lipid peroxidation and lower redox state of glutathione (Kučera *et al.* 2014). Although we did not observe significant differences in ROS generation and lipid peroxidation between control groups of lean and steatotic hepatocytes in this experiment, ROS production and MDA concentration in cell lysate were almost 1.4-fold and 2-fold higher in control fatty cells than in control non-steatotic hepatocytes. These alterations may predispose steatotic hepatocytes to increased susceptibility to oxidative damage.

Mitochondrial dysfunction and mitochondrial permeability transition (MPT) were reported to participate in hepatotoxicity of GalN *in vitro* and *in vivo* (Quintero *et al.* 2002, Soriano *et al.* 2004). In this work, we estimated MMP by a fluorescent probe JC-1 and calculated hepatocytes containing energized mitochondria. Fatty cells exposed to GalN were significantly more susceptible to loss of MMP than lean hepatocytes. Higher sensitivity of mitochondria to loss of MMP was previously proven in steatotic hepatocytes exposed to exogenous source of oxidative stress (Kučera *et al.* 2014). Overproduction of ROS is a known inducer of mitochondrial dysfunction and MPT (Halestrap *et al.* 2002). MPT in hepatocytes can be also induced by TNF $\alpha$  (Hatano *et al.* 2000). Whereas we did not observe any differences in TNF $\alpha$  between lean and steatotic hepatocytes exposed to GalN, markers of oxidative stress were significantly higher in steatotic hepatocytes treated with GalN.

In conclusion, this study shows that steatotic hepatocytes are more susceptible to the toxic effect of GalN. This sensitivity may result from higher GalN-induced ROS generation and lipid peroxidation and higher susceptibility of mitochondria to loss of mitochondrial membrane potential in steatotic hepatocytes. In our experimental arrangement, apoptosis does not seem to participate considerably on hepatotoxic action of GalN in either group of hepatocytes. Although it is not possible to simply extrapolate the outcomes from non-human experiments *in vivo* or *in vitro* to clinical practice, the results of our study and our previous works (Garnol et al. 2014, Kucera et al. 2011b, Kucera et al. 2012a, Kucera et al. 2012b, Kučera et al. 2014) indicate that simple fatty transformation of hepatocytes is not only benign morphological change but predisposes hepatocytes to enhanced susceptibility to hepatotoxins of different mechanisms of action.

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## Conflict of Interest

There is no conflict of interest.

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### Figure legends:

Figure 1 - LDH leakage (A) and activities of caspase 3 (B), caspase 8 (C) and caspase 9 (D) in cell lysate after 24-hour incubation of lean and steatotic hepatocytes without (NH\_C and SH\_C, respectively) or with GalN (NH\_GalN and SH\_GalN, respectively). The values are means $\pm$ SD of 3 (for A, C, D, n = 16 - 18) or 4 (for B, n = 23 - 24) independent experiments. Data are expressed in % of NH\_C (B, C and D). \* p<0.05 and \*\*\* p<0.001 vs NH\_C; # p<0.05 and ### p<0.001 vs SH\_C; \$ p<0.05 and \$\$\$ p<0.001 vs NH\_GalN of appropriate concentration of GalN.

Figure 2 – Production of ROS (A) and concentration of MDA in cell lysate (B) after 24-hour incubation of lean and steatotic hepatocytes without (NH\_C and SH\_C, respectively) or with GalN (NH\_GalN and SH\_GalN, respectively). The values are means $\pm$ SD of 3 (for B, n = 18) or 4 (for A, n = 22-24) independent experiments. Data are expressed in % of NH\_C. \* p<0.05 and \*\*\* p<0.001 vs NH\_C; ## p<0.01 and ### p<0.001 vs SH\_C; \$ p<0.05 and \$\$ p<0.01 vs NH\_GalN of appropriate concentration of GalN.

Figure 3 – Concentration of TNF $\alpha$  in culture medium after 24-hour incubation of lean and steatotic hepatocytes without (NH\_C and SH\_C, respectively) or with GalN (NH\_GalN and SH\_GalN, respectively). The values are means $\pm$ SD of 4 independent experiments (n = 22 - 24). Data are expressed in % of NH\_C. \*\*\* p<0.001 vs NH\_C; ### p<0.001 vs SH\_C.

Figure 4 – Percentage of attached hepatocytes containing energized mitochondria after 24-hour incubation of lean and steatotic hepatocytes without (NH\_C and SH\_C, respectively) or

with GalN (NH\_GalN and SH\_GalN, respectively). The values are means $\pm$ SD of 3 independent experiments (n = 18 - 28). \* p<0.05 and \*\*\* p<0.001 vs NH\_C; # p<0.05 and ### p<0.001 vs SH\_C; \$ p<0.05 and \$\$ p<0.01 vs NH\_GalN of appropriate concentration of GalN.

Figure 5 – Visualization of changes in mitochondrial membrane potential using mitochondria-specific fluorescent probe JC-1. Mitochondria with intact membrane potential concentrate JC-1 and exert orange fluorescence whereas de-energized mitochondria emit green fluorescence. Microphotographs of non-steatotic and fatty rat hepatocytes cultured in William's E medium for 24 hours without (A and B, respectively) or with 20 mM GalN (C and D, respectively). Objective magnification 40x, bar 50  $\mu$ m.

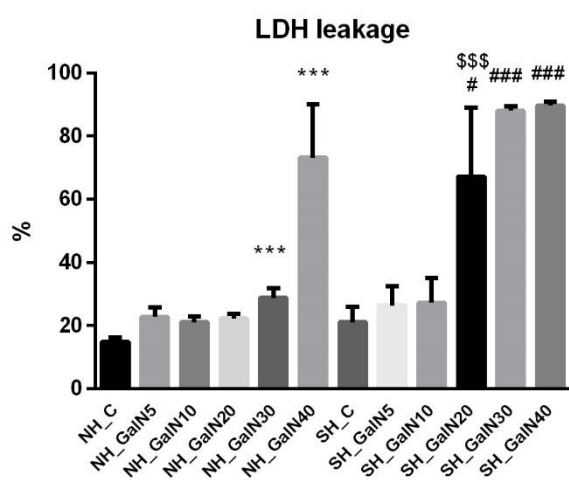
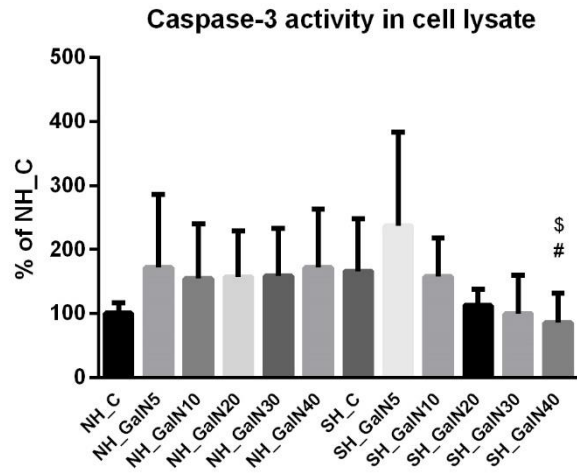
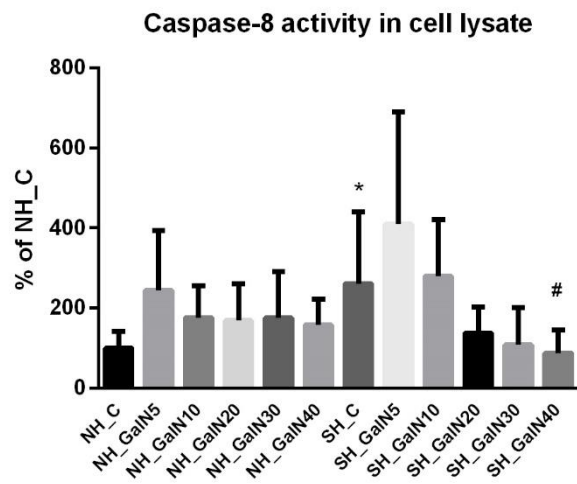


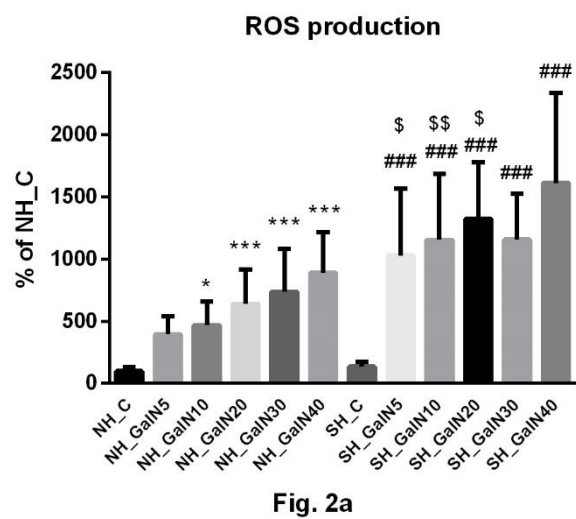
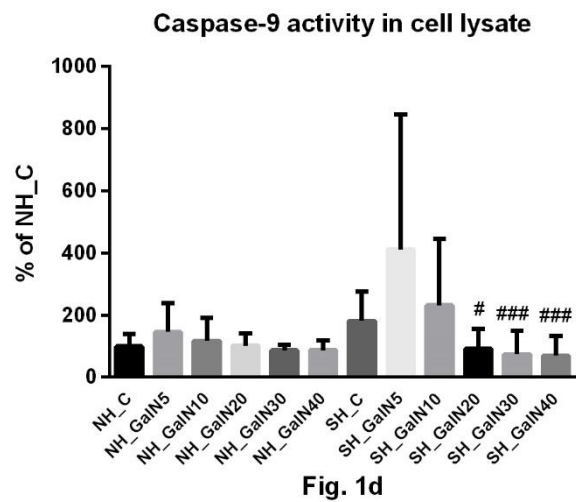
Fig. 1a



**Fig. 1b**



**Fig. 1c**



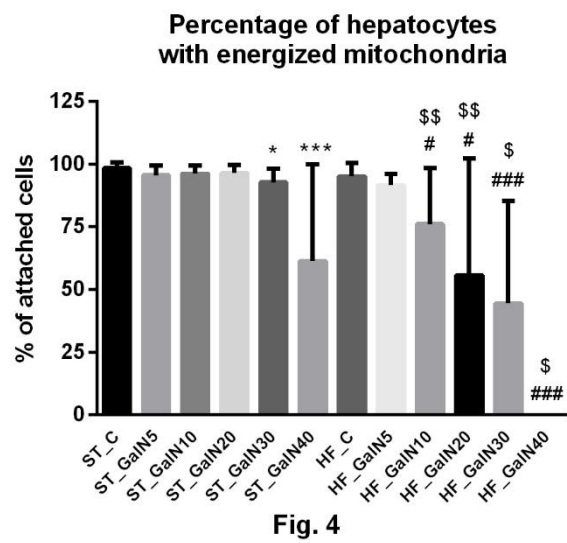
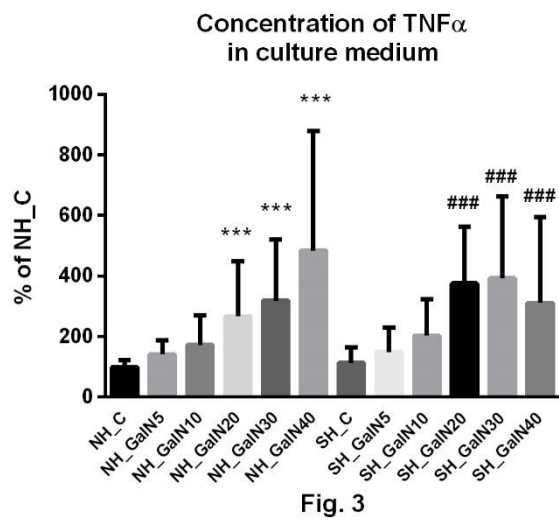
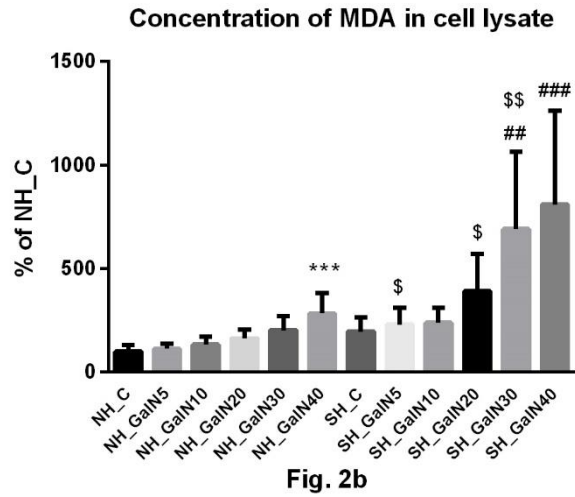


Fig5a

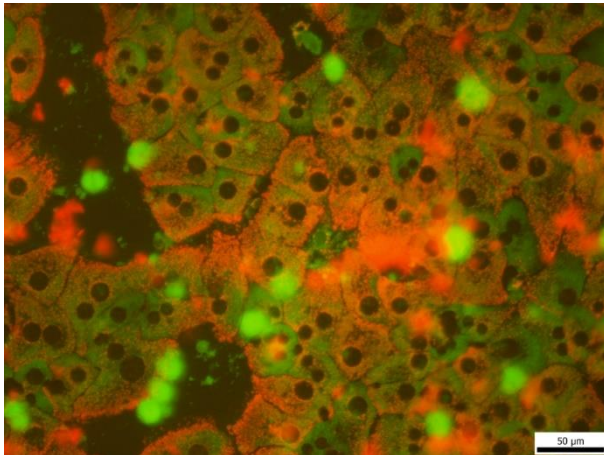


Fig 5b

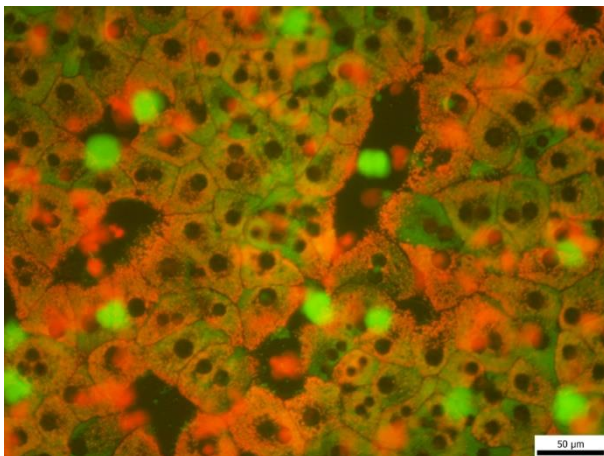


Fig 5c

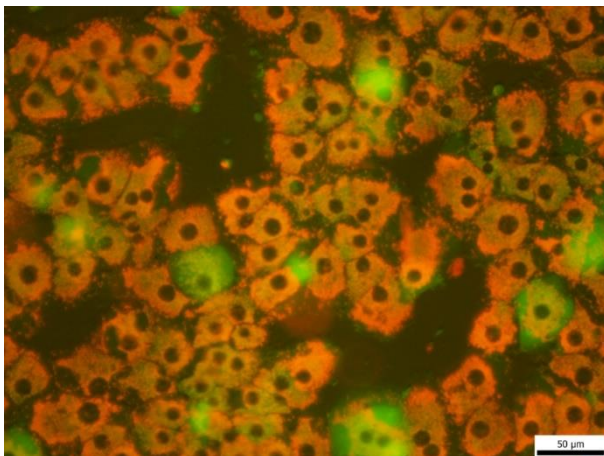




Fig 5d

