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Methods Article

METHODS ARTICLE

# Preparation of fatty acid solutions exerts significant impact on experimental outcomes in cell culture models of lipotoxicity

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## Abstract

Free fatty acids are essentially involved in the pathogenesis of chronic diseases such as diabetes mellitus, non-alcoholic fatty liver disease, and cardiovascular disease. They promote mitochondrial dysfunction, oxidative stress, respiratory chain uncoupling, and endoplasmic reticulum stress and modulate stress-sensitive pathways. These detrimental biological effects summarized as lipotoxicity mainly depend on fatty acid carbon chain length, degree of unsaturation, concentration, and treatment time. Preparation of fatty acid solutions involves dissolving and complexing. Solvent toxicity and concentration, amount of bovine serum albumin (BSA), and ratio of albumin to fatty acids can vary significantly between equal concentrations, mediating considerable harmful effects and/or interference with certain assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Herein, we studied the impact of commonly used solvents ethanol and dimethyl sulfoxide and varying concentrations of BSA directly and in solution with oleic acid on MTT to formazan conversion, adenosine triphosphate level, and insulin content and secretion of murine β-cell line MIN6 employing different treatment duration. Our data show that experimental outcomes and assay readouts can be significantly affected by mere preparation of fatty acid solutions and should thus be carefully considered and described in detail to ensure comparability and distinct evaluation of data.

*Keywords:* free fatty acid; oleic acid; lipotoxicity; beta cell; BCRJ Cat# 0293; RRID: CVCL_0431; diabetes mellitus; sample preparation

## Introduction

### Lipotoxicity

Visceral obesity is one of the major causes of insulin resistance and development of type 2 diabetes mellitus (T2DM) [1, 2]. Abundance of energy fuels insulin resistance and leads to dyslipidemia marked by pathological increase of free fatty acids (FFA). Detrimental effects mediated by FFA were first described in 1994 and termed as “lipotoxicity” [3]. Interactions between FFA and cells are complex and comprise multiple pathways eventually interfering with lipid metabolism. Dysregulation of uptake, utilization, and/or storage of triglycerides and FFA [4, 5] leads to accumulation of bioactive molecules such as acyl-CoA and ceramides [6, 7] triggering pathways including mitogen-activated protein kinase (MAPK), extracellular-signal-regulated kinase (ERK), sterol regulatory element binding protein-1c (SREBP-1c), and uncoupling protein 2 (UCP2) or promoting release of intracellular Ca storages [8–11]. Apart from diabetes

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research, lipotoxicity plays a role in the pathogenesis of non-alcoholic fatty liver disease [12], cardiovascular disease [13], and apoptosis promoted by cytochrome C release [14]. Therefore, establishment of standardized protocols for *in vitro* models with defined FFA as lipid stressors is required for the investigation of underlying mechanisms.

Preparation of FFA solutions applicable for immortalized or primary cell lines comprises dissolving and complexing FFA. According to our review of over 100 articles about lipotoxic effects on mitochondria in pancreatic β-cells, protocols frequently do not contain essential information on how FFA solutions were prepared [15]. Since equally concentrated FFA solutions can be composed distinctly differently and thus have significant impact on experimental outcomes, we aim to have a closer look at the respective experimental parameters.

### Type of FFA and solubility

Each FFA is characterized by physicochemical properties including solubility by carbon chain length and number of double bonds. Frequently used FFA in diabetes research are long-chain fatty acids namely palmitic (PA) and oleic acid (OA) representing the most abundant saturated and unsaturated FFA in T2DM, respectively [16]. For solubilizing FFA and preparing a high molecular stock solution, most frequently employed protocols include ethanol (26%), saponification (12%), dimethyl sulfoxide (DMSO) (4%), and methanol (3%), while 55% of articles do not specify the substance used [15]. However, solubility of FFA and respective composition of solutions differ significantly among solvents. Solubility of PA in DMSO is 80 mM compared with 350 mM for OA (Cayman Chemical, Ann Arbor, MI, USA). In contrast, there seems to be no relevant difference in ethanol solubility for preparing stock solutions. A concentration as high as 1800 mM for PA/OA was achieved with ethanol [17]. While toxicity of DMSO in different models is lower than ethanol at same concentrations [18], the amount of ethanol in the final FFA concentration can be lowered to a greater extent because of higher dilution factors. Therefore, ethanol allows studying higher FFA concentrations with low levels of solvent. Methanol is rarely used as solvent, but seems to be less toxic in human and rat cell lines, while solubility is comparable to ethanol [19]. In contrast, saponification doesn't yield harmful solvent effects as sodium salts of FFA are created by usage of NaOH or NaCl. To saponify FFA, higher temperatures have to be used.

### Bovine serum albumin

Fraction V fatty acid-free bovine serum albumin (BSA) is used to complex FFA in aqueous conditions like cell culture media. FFA are physiologically complexed to albumin [20]. The biopolymer BSA has different functions depending on respective experimental settings. In cellular systems, cholesterol [21] and opioid binding characteristics are described [22]. Thiol groups of BSA can act as antioxidant [23] and inhibition of angiotensin-converting enzyme was described in a cell model of hypertension for human serum albumin [24]. It is used as a blocking agent for methods based on antibody interactions and as protein standard. Its complexation ability can be used for complexing metals or FFA. As BSA carries FFA it is involved in energy metabolism and composition of biomembranes. BSA will increase FFA bioavailability in organisms [25]. For complexing FFA, BSA can be added into cell culture media and after gently mixing FFA stock solution is added. FFA can be protected from oxidation by overlaying with nitrogen and will complex to BSA by shaking for several hours at 37°C. After sterile filtration, FFA media can be stored at −20°C. The amount of BSA will determine the final molar FFA:BSA ratio. The higher the FFA:BSA ratio the greater is the amount of FFA unbound to BSA. FFA-mediated detrimental effects on cellular functions are crucially determined by the amount of unbound FFA [26]. It is suggested that a 5:1 ratio is suitable to mimic pathological conditions and should not be exceeded [27]. When a stable FFA:BSA ratio is maintained, the amount of BSA will increment with increasing concentrations of FFA interfering with substrates employed in experimental settings like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [27, 28]. Conversion of MTT to formazan assesses the reduction potential of a cell, that is the biofunctionality of dehydrogenase enzymes depending on reducing compounds to drive cellular energetics [29]. The assay can distinguish living from dead cells, as active mitochondria are required to perform this reaction. A high conversion rate of MTT is taken as parameter for metabolically active cells with high capacity of reduction equivalents. By examining various control conditions, we have observed that this reaction can be induced by BSA.

### Aim of research

We studied the impact of ethanol and DMSO, BSA, treatment duration, and differentially prepared OA solutions on MTT to formazan conversion, adenosine triphosphate (ATP) level, and insulin content and secretion of murine β-cell line MIN6.

## Material and methods

### Cell culture

Mouse insulinoma (MIN6) cells (BCRJ Cat# 0293, RRID: CVCL_0431) were obtained from Prof. Lenzen of Hannover Medical School, Hannover, Germany [30]. Cells of passages 50–60 were cultured in an incubator at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) enriched with 25 mM glucose and 1 mM pyruvate (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 16% v/v heat inactivated fetal bovine serum (Biowest, Riverside, MO, USA), 80 U/mL penicillin/streptomycin (Gibco, Thermo Fisher Scientific), and 50 μM 2-mercaptoethanol (Gibco, Thermo Fisher Scientific). Change of culture media was performed every 2–3 days. After reaching a confluency of 70–80%, PBS (Lonza, Basel, Switzerland) washed cells were collected by trypsinization with 0.05% Trypsin-EDTA (Gibco, Thermo Fisher Scientific). After centrifugation at 1200 rpm for 4 min, supernatant was removed and MIN6 cells were resuspended in fresh DMEM. Cell count was determined using a Neubauer counting chamber (Brand, Wertheim, Germany) after staining with Trypan blue (Sigma-Aldrich, St. Louis, MO, USA).

### Preparation of treatment solutions

#### *BSA, DMSO, ethanol*

Ethanol (purity >99.8%, Sigma, Munich, Germany), DMSO (purity >99.5%, Sigma), or fraction V fatty acid free BSA (SERVA Electrophoresis, Heidelberg, Germany) was added to cell culture DMEM. Prior to treatment, solutions containing ethanol or DMSO were mixed properly for several seconds. BSA was dissolved overnight on a shaker at 37°C, followed by sterile filtration (0.22 μm).

#### *FFA solution*

OA (purity >99%, Enzo Life Sciences, New York, USA) was dissolved in ethanol (purity >99.8%, Sigma) to prepare stock solutions of 150, 225, 450, and 900 mM. Stock solutions were diluted

1:300 with DMEM complexed with fraction V fatty acid-free BSA (SERVA Electrophoresis, Heidelberg, Germany) to prepare FFA concentrations of 0.5, 0.75, 1.5, and 3 mM. The molar FFA:BSA ratio was 5:1 and ethanol concentration was 0.23–0.32% w/w. The prepared FFA media were overlayed with nitrogen (Linde, Dublin, Ireland) and placed on a shaker overnight at 37°C. After sterile filtration (0.22 μm), stock solutions were stored at −20°C. Control media contained matching amounts of ethanol and BSA. For comparison of solvent effects, a 150 mM OA stock solution and control medium were prepared with DMSO (purity >99.5%, Sigma). Stock solution was diluted to generate 1.5 mM OA media. A suggested standard protocol for preparation of FFA solutions is given in the Supplementary Data.

### MTT assay

MTT assay was chosen for evaluation of cell functionality. After $1 * 10^4$ MIN6 cells in 200 μL DMEM had attached to a 96-well plate (Greiner Bio-One, Kremsmünster, Austria) for 24 h, DMEM was replaced by fresh DMEM, control, or treatment medium, respectively, for 24 or 120 h (5 days). In case of 120 h treatment medium was changed after 0 (attachment time), 48, and 96 h. Ten milligrams of MTT (purity >98%, Abcam, Cambridge, UK) was dissolved in 5 mL PBS (c = 2 mg/mL). After sterile filtration (0.22 μm), 50 μL of media was replaced by MTT solution, protected from light. Samples were incubated at 37°C for 4 h. Afterward the entire medium was replaced by 200 μL DMSO. Formazan crystals dissolved for 15 min on a plate shaker and absorption values were measured at 570 and 620 nm (background) by a Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). Absorption value of DMEM without MIN6 cells was subtracted as a blank value.

### ELISA

$4 * 10^5$ MIN6 cells in 3 mL DMEM were seeded into a six-well plate (Greiner Bio-One, Kremsmünster, Austria). After attaching to the plate for 24 h, cells were exposed to 1.5 mM OA for 24 h. One milliliter of culture medium was collected and centrifuged at 2000 rpm for 5 min at 4°C. Supernatant was collected for further analysis. PBS-washed cells were lysed with 300 μL of NP-40 buffer (10% aqueous solution, Merck Millipore, Burlington, MA, USA) supplemented with 3% v/v protease inhibitor cocktail 100× (Halt, Thermo Fisher Scientific, Waltham, MA, USA) for 20 min at 4°C. After centrifugation at 12,000 rpm for 15 min at 4°C, supernatant of cell lysate was collected. Insulin content of cell lysate and culture medium was measured by direct sandwich ELISA (DRG Diagnostics, Marburg, Germany). An aliquot of lysate samples was analyzed for total protein by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) to normalize insulin results to total protein [31]. In total, 0.1–1 mg/mL BSA was used as standard.

### ATP bioluminescence

$5 * 10^4$ MIN6 cells were seeded into a 96 luminescence-well plate (Corning Inc., Corning, NY, USA) and attached for 24 h. After attaching solvent treatment was performed for 24 h. ATP level was determined by adding a NaOH lysis buffer and a solution containing luciferase, luciferin, and co-substrates. The assay was performed according to manufacturer's instructions (PerkinElmer, Waltham, MA, USA). Luminescence was measured using a Multimode Microplate Reader (CLARIOstar, BMG LABTECH, Ortenberg, Germany).

### Statistical analysis

Statistical analysis was performed by GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) using one- or two-way ANOVA with Tukey's multiple comparisons test. Data are represented as mean ± SD. A $P$-value <0.05 was considered significant and marked with *. $P < 0.01$, <0.001, and <0.0001 were marked with **, ***, and ****, respectively.

## Results

### BSA increases MIN6 cell-dependent MTT conversion to formazan

After 24 h cultivation of MIN6 cells with 0.5–12.5% w/w BSA, the conversion of MTT to formazan was increased by BSA (Fig. 1A). This effect was observable up to a maximum concentration of 6% BSA increasing conversion by ∼60%. After exceeding a concentration of 12.5% a negative effect set in. Cultivation with 12.5% BSA led to an overall increased conversion of ∼25% compared with DMEM-treated cells. Treatment with 5% w/w ethanol was chosen as toxic treatment decreasing reducing activity measured by MTT conversion by ∼60%. In contrast, prolonged cultivation for 5 days led to a non-significant increase with 1% and 3% BSA, whereas 6–10% BSA inhibited MTT to formazan conversion by up to 50% (Fig. 1B). Absorption levels with 6%, 8%, and 10% BSA were below controls after 5 days culture.

### BSA increases MIN6 cell-dependent and independent MTT conversion to formazan

The impact of BSA concentrations ranging from 1% to 10% w/w on MTT assay with (Fig. 2A) or without (Fig. 2B) MIN6 cells was studied. Conversion of MTT to formazan in medium containing BSA without cells was dose-dependently rising. Relative enhancement was significant for higher concentrations of BSA while the absolute increase in absorption was minimal.

To take into account the MIN6-independent effect of BSA absorption readings of solutions without cells was subtracted from corresponding cell samples. This normalization resulted in a 25% decrease in absorption for 10% BSA when compared with lower concentrations (Fig. 3) in contrast to a 17% decrease without normalization (Fig. 2A).

### Increased treatment duration intensifies lipotoxic effects and MTT to formazan conversion

To study the effects of treatment duration, MIN6 cells were exposed to 0.5–3 mM OA with corresponding control solutions for 24 h (Fig. 4A) and 5 days (Fig. 4B), respectively. For both setups, a dose-dependent toxic effect was observed. It was more pronounced after 5 days of treatment. Prolonged culture doubled absorption readouts in comparison with 24 h cultivation.

### DMSO and ethanol significantly affect MTT to formazan conversion in a dose-dependent manner

DMSO treatment of MIN6 cells for 24 h showed a negative effect on MTT to formazan conversion for 4% w/w, whereas 0.5–2% w/w increased optical density slightly (Fig. 5). 1–4% w/w ethanol mediated a more pronounced reduction of MTT conversion, while 0.5% and 1% did not show a significant difference in optical density after 24 h.

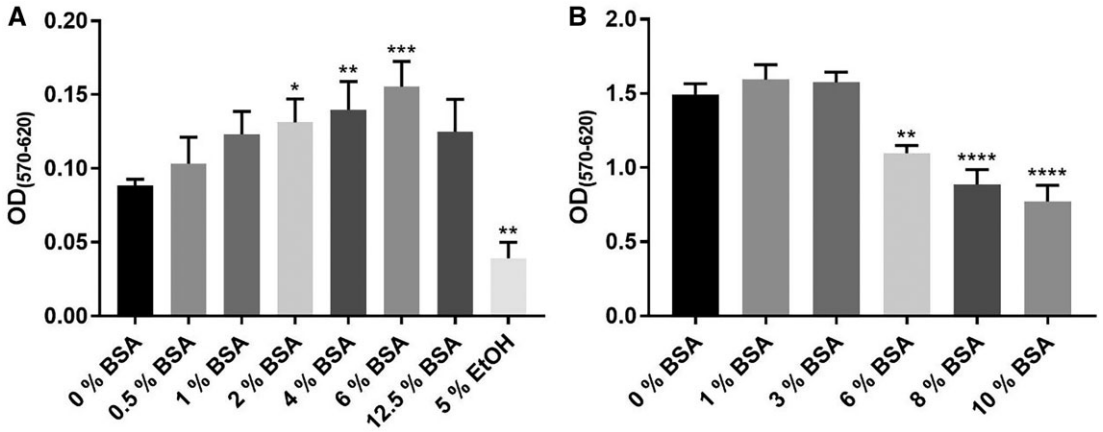


**Figure 1:** Optical density obtained from MTT assay after (**A**) 24 h treatment of MIN6 cells with different BSA concentrations and ethanol as toxic treatment ($n = 4$) and (**B**) 120 h (5 days) treatment of MIN6 cells with different BSA concentrations ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared with 0% BSA.

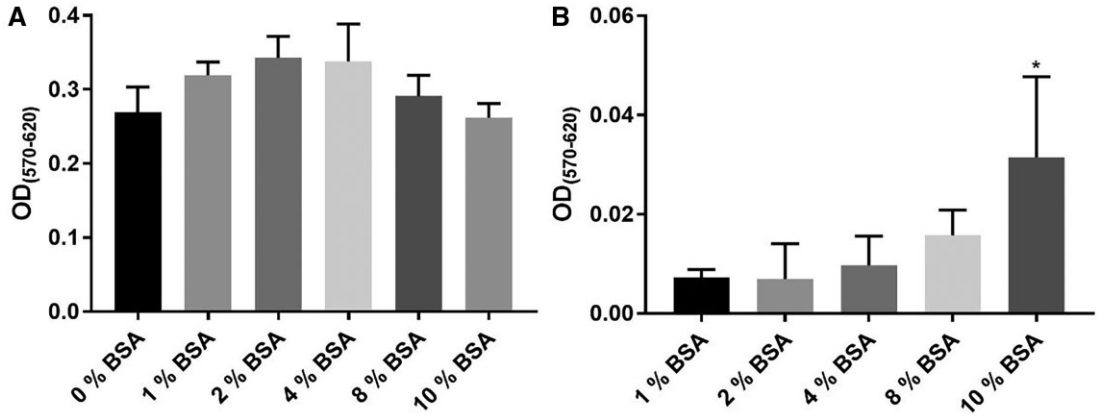


**Figure 2:** Optical density obtained from MTT assay (**A**) after treatment of MIN6 cells with different BSA concentrations for 24 h ($n = 3$) and (**B**) for different BSA concentrations without MIN6 cells ($n = 3$). *$P < 0.05$ compared with (B) 1% BSA.

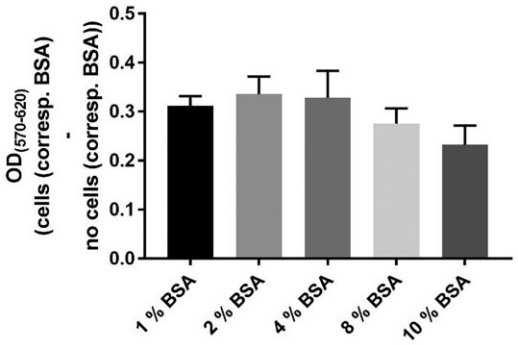


**Figure 3:** Normalized optical density after 24 h treatment with BSA ($n = 3$). Readings of solutions without cells were subtracted from corresponding cell samples.

### 24 h ethanol treatment decreases ATP content of MIN6 cells

Treatment with ethanol for 24 h decreased intracellular ATP level (Fig. 6). The effect was more pronounced by higher ethanol concentration and significant for 0.45% w/w and 0.9% w/w. Treatment with 0.225% led to a non-significant decrease of 30% while 0.9% decreased ATP by 50%.

### Ethanol-prepared FFA exhibit greater impairment of insulin than DMSO-prepared FFA

For comparison of solvent effect on insulin synthesis, 1.5 mM OA was dissolved in ethanol (final concentration = 0.45% w/w) or DMSO (final concentration = 0.96% w/w). 1.5 mM OA dissolved in ethanol mediated a ~90% decrease of insulin concentration in cell lysate (Fig. 7A) and in culture media (Fig. 7B), indicating abated insulin secretion. Preparation with DMSO diminished lysate insulin by ~40% and secreted insulin in medium by ~85%. The presence of DMSO did not impair insulin production to such extent as the presence of ethanol did despite its higher concentration.

## Discussion

It is acknowledged that the effects of FFA treatment are essentially dependent on carbon chain length, degree of unsaturation, and concentration of the respective fatty acid as well as treatment duration [15]. However, preparation of FFA can vary significantly, and little is known about a possible impact on assay readouts and experimental outcomes.

### Bovine serum albumin

BSA initially enhanced conversion of MTT to formazan in a concentration-dependent manner in MIN6 culture before a

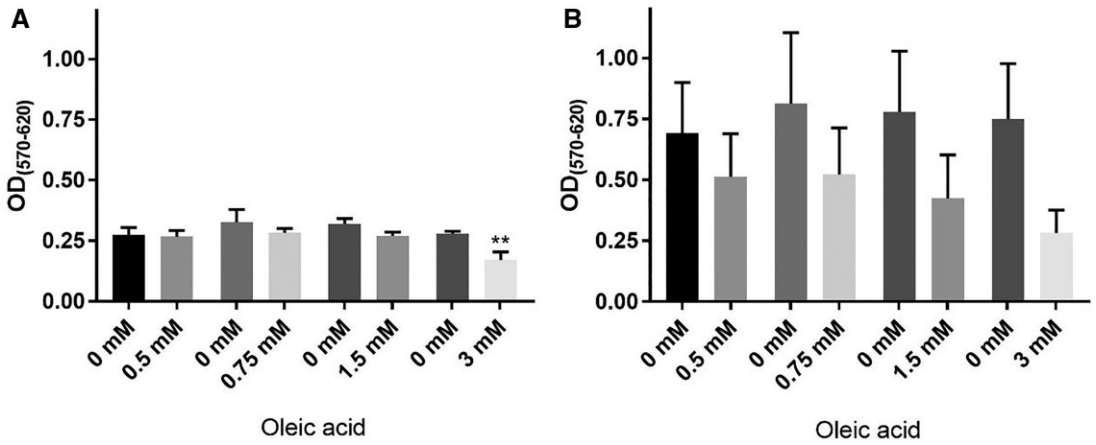


**Figure 4:** Optical density obtained from MTT assay of MIN6 cells after (**A**) 24 h and (**B**) 120 h (5 days) treatment duration with different concentrations of OA or corresponding controls ($n = 3$). $^{**}P < 0.01$ compared with respective control treatment without fatty acid.

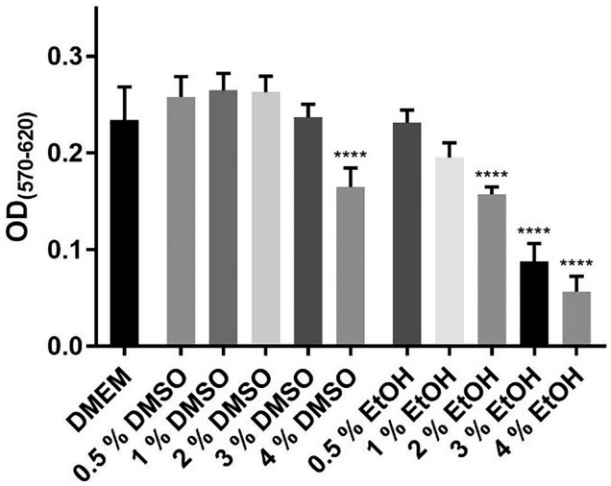


**Figure 5:** Optical density obtained from MTT assay of MIN6 cells treated for 24 h with solvents DMSO and ethanol ($n = 5$). $^{****}P < 0.0001$ compared with cell culture with DMEM.

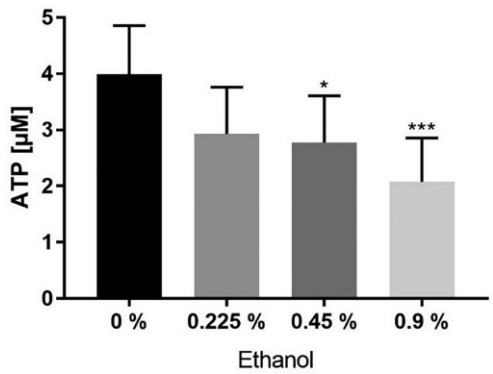


**Figure 6:** ATP level of MIN6 cells after 24 h treatment with ethanol ($n = 8$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with 0% ethanol.

decrease was observed at higher concentrations. In a setup without MIN6 cells, conversion increased steadily. The impact of BSA on experimental outcomes has not been studied extensively. It is unclear whether it is caused by an interference with conversion of MTT to formazan or altered cytobiological function. Possible mechanisms underlying a cell-dependent effect are growth factors; carrier function for small molecules; binding of toxic substances; or the supplementation of cell culture media with essential compounds like lipids, amino acids, and metal ions [32, 33]. As Huang *et al.* [34] have demonstrated BSA significantly reduces hydroxyl groups taking effect on antibody reactions. Secretion of IL-1β and TNF-α was promoted by BSA in microglial cells [35]. In brain tissue, BSA increased oxidation in mitochondria particularly by activation of succinate dehydrogenase [36]. Ponte *et al.* [37] demonstrated that BSA can modulate muscle cell response to calcium release of the sarcoplasmic reticulum. Remarkably, high concentrations of BSA led to lower conversion of MTT in MIN6 samples. General proteotoxic effects [38] might explain this decrease. By assumption those high concentrations of 6% or more with impairing effects are not used in regular cell culture work. For cell-independent effects, a direct effect of BSA on MTT was strikingly evident. Although absolute absorption levels were only affected marginally, normalization with corresponding cell-independent BSA controls can be a helpful tool to clarify the cellular effects, especially in models with small differences between samples. A major concern is that cellular viability as determined by MTT assay is overestimated if BSA in control media is not adjusted. Maximum effect in our experiments was +50% in MTT to formazan conversion. It should also be considered that absolute absorption levels are time-dependent. After 5 days of culture, optical density approximately doubled when compared with 24 h (Fig. 4). So far it is not known if prolonged treatment with BSA containing media has a greater effect on cell-independent MTT reaction. Judging from our data, a cell-dependent and -independent effect might be possible. By rough estimation, 1% of BSA in DMEM increased MIN6 viability by 10%. By assuming a 5:1 ratio, most used models investigating 0.5 mM FFA apply 0.1 mM BSA representing 0.66% BSA. Exposition against low molecular FFA concentration should physiologically only lead to a slightly reduced viability [39–41]. Therefore, BSA exerts a relevant impact on MTT outcome when compared with its lipotoxic effect and might conceal FFA-mediated effects. In dosages mediating a significant level of lipotoxicity, this impact becomes negligible. The significance of these effects is highly depending on the experimental setup. If multiple concentrations are tested, greater differences between employed amounts of BSA occur. According to our previous work, this is the case for approximately 21% of articles on lipotoxicity in diabetic models [15]. To be able to assess the impact of BSA, an exact

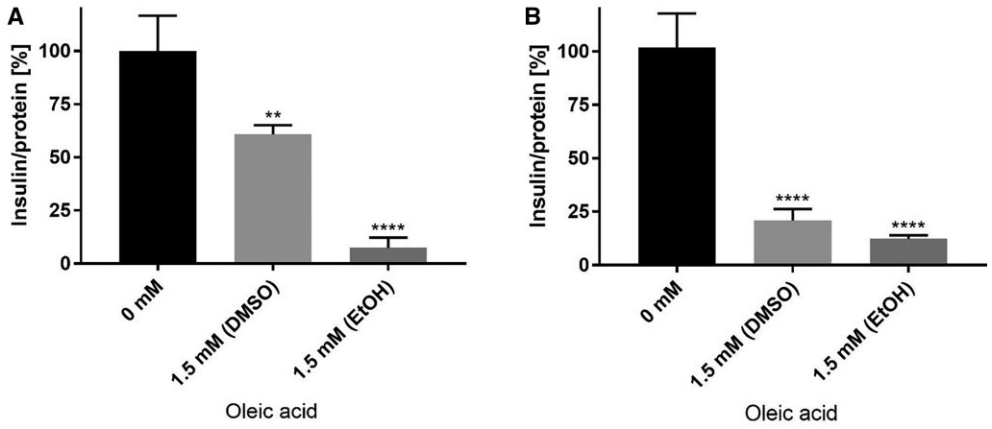


**Figure 7:** Insulin amount of (**A**) MIN6 cell lysate and (**B**) culture medium after 24 h treatment with 1.5 mM OA prepared with DMSO or ethanol ($n = 4$). $^{**}P < 0.01$, $^{****}P < 0.0001$ compared with 0 mM.

methodological description is necessary. A concentration range of 0.5–2 mM FFA would result in a BSA range of 0.1–0.4 mM representing 0.66–2.77% BSA. This could lead to a change of viability of 20%. Therefore, it is necessary to prepare individual control solutions for all treatment conditions in MTT assays. In diabetes research, outcomes also depend on FFA concentration as concentrations below 0.1 mM exert positive effects on insulin secretion as opposed to concentrations of 0.5 mM and above. Underlying mechanisms involve promoted insulin secretion by depletion of Ca storages induced by interaction of peroxisome proliferator-activated receptor γ (PPARγ) and G protein-coupled receptor 40 (GPR40), an effect which is especially seen after acute treatment [42–44]. Physiological levels of FFA can be further used for energy production increasing insulin secretion by elevated ATP levels [45]. By chronic exposure, energy production is impaired by SREBP-1c-induced uncoupling of respiratory chain complexes by UCP2. Beside possible interactions of BSA, both FFA concentration and treatment duration can change experimental outcomes. Also, molar ratio is a decisive parameter in lipotoxic research. The chosen ratio of FFA:BSA can generally determine lipotoxic effects throughout different assays. It was shown that mere changes can induce apoptotic effects [26] which are prevented by lower molecular ratios [27]. These changes in toxicity can also be apparent due to different preparations of BSA. The amount of albumin can be affected by using commercially available FFA-free albumin or charcoal-absorbed BSA which can be used for removing bovine FFA, potentially altering the outcome of certain assays. There are vast differences in ratios employed throughout literature ranging from 74:1 [46] to 1:3 [47] or 1:5 [44]. To achieve the same FFA concentration of 0.5 mM, different protocols give markedly divergent concentrations of BSA like 0.045% [46], 10% [47], or 16.6% [44]. As 54% of reviewed articles neither state the amount of BSA nor the FFA:BSA ratio, it is doubtful that the general impact of BSA is commonly taken into account. It would be helpful to state that BSA and solvents were used as controls and their effects on cell viability were evaluated [48]. The matter is further complicated by the enrichment of cell culture medium by BSA-containing serum.

### Solvents

Direct toxic effects of solvents for FFA preparation have to be considered carefully. Possible solvents have differential toxic impact on cellular systems as shown by MTT and insulin ELISA. DMSO seems to be less toxic for equimolar concentrations compared with ethanol. Due to restrictions in solubility especially the use of highly concentrated PA requires high concentrations of DMSO. Systematic evaluations of common solvents in organisms serve as reference [18]. Embryotoxic effects commence at ethanol concentrations above 1%. Equal concentrations led to negative effects in performed MTT assays (Fig. 5). It can't be excluded that metabolic restrictions also occur at lower concentrations.

The majority of articles does not provide the used solvent and/or different concentrations are not discriminated in terms of solvent concentration. When multiple concentrations of FFA are used, a detailed description of respective preparations favoring the preparation of individual stock solutions would be desirable. A single stock solution applied for a range of concentrations leads to decreased levels of solvents at higher dilution and vice versa. Lipotoxic and solvent-induced effects might thus be difficult to be distinguished. Similarly, solvent controls should be prepared for each concentration. This becomes especially relevant for articles studying higher FFA concentrations due to solubility characteristics. As 0.5 mM is the most frequently chosen concentration applied, few articles are exceeding the concentration. Nine out of 132 articles reviewed for FFA-mediated effects on mitochondrial function [15] employed a concentration between 1 and 2 mM. Three working groups gave sufficient information to calculate final ethanol solution which was lower than 0.31%. Two articles used saponification. There was not sufficient or no data at all on preparation of FFA solutions in the remaining articles. Without these information, respective solvent-mediated effects cannot be considered. As an example, a 1 mM OA solution prepared from a 300 mM stock solution will contain a final ethanol concentration of 0.3%, while using a 100 mM stock solution will result in 0.97% ethanol, potentially exerting significant toxic impact on sensitive cell lines or assays as demonstrated by our data (Fig. 5–7).

## Limitations

Our aim was to generate a systematic approach to study relevant parameters in FFA preparation employing a widely used β-cell model. Data presented herein have been collected from 2019 to 2021 and were consistent with previously mostly unpublished data gathered by multiple researchers from our group during the last 5 years. However, there are limitations to this study. Only single effectors were tested, leaving the question if

and how respective concentrations take impact on experimental outcomes once combined in the final treatment solution. We mainly focused on MTT assay as predictor of cellular function. Future studies should thus evaluate the impact of FFA solution preparation on a broader range of assays. Our data further revealed that the quantitative impact on experimental outcomes depends on the respective assay. Treatment with 1% ethanol only changed MTT results slightly whereas cellular ATP content was diminished by 50% and exposure to 1.5 mM OA led to a 20% decrease in MTT reaction but a 90% decrease in insulin ELISA readout. The impact of the effectors employed in this study on mere assay results must thus be carefully delineated from their biological effects and considered for interpretation of experimental outcomes, especially regarding potential implications for *in vivo* studies. Comparison of albumin levels and experimental parameters to mimic physiological conditions is difficult. Level of human serum albumin ranges from 550 to 700 μM in healthy individuals [49] with very limited data on the effects of BSA. Respective studies involve various experimental models and concentrations applying 0.7–30 μM [35–37] while BSA concentration and/or FFA:albumin molar ratio in diabetic research are often not stated [15, 27]. Applied BSA concentration in the current study comprised 100–600 μM correlating with (sub-)physiological levels. However, medical conditions such as diabetes mellitus [50] or liver disease [51] can significantly affect serum albumin levels. Drawing conclusions from our data on the significance for *in vivo* assays does thus not seem feasible.

## Conclusion

Our data demonstrate that preparation of FFA solutions can exert significant impact on experimental outcomes when studying lipotoxic effects. Effects of utilized solvents, amount of BSA, molar relation of FFA to BSA, treatment time, and choice of controls can exponentiate or counteract each other to a great extent. Popular assays used for estimating cell viability like MTT to formazan conversion are susceptible to BSA. Solvents in general have detrimental effects on cells. It is difficult to predict and analyze the outcomes of these effects, especially since information on preparation of FFA solutions is frequently omitted or incomplete in literature. We recommend that researchers give respective information to allow for comparability and appropriate interpretation of data. A suggested protocol is given in the Supplementary Data.

## Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

## Data availability

All data are included within this manuscript and its Supplementary Files.

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## Author contributions

A.R.: conceptualization, methodology, acquisition, analysis, interpretation of data, original draft preparation, and visualization. D.R.: critical review and editing. T.L.: conceptualization, critical review, and editing. S.F.P.: conceptualization, critical review, editing, and supervision. All authors have read and agreed to the published version of the manuscript.

## Conflict of interest statement

The authors declare no conflicts of interest.

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