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Albumin promotes proliferation of G1 arrested serum starved hepatocellular carcinoma cells

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Albumin is the most abundant plasma protein and functions as a transport molecule that continuously interacts with various cell types. Because of these properties, albumin has been exploited by the pharmaceutical industry to improve drug delivery to target cells. Understanding the immediate effects of albumin on cells is however limited. The cell interacting properties and pharmaceutical applications of albumin incentivises continual research into the immediate effects of albumin on cells. The HepG2/C3A hepatocellular carcinoma cell line is used as a model for studying cancer pathology as well as liver biosynthesis and cellular responses to drugs. Here we investigated the direct effect of purified albumin on HepG2/C3A cell proliferation in the absence of serum, growth factors and other serum originating albumin bound molecules. We demonstrated that serum starvation causes HepG2/C3A cell numbers to stagnate, whereas inclusion of albumin allows an increase in cell numbers. Cell cycle analysis demonstrated that the percentage of cells in G1 phase during serum starvation was reduced by the inclusion of albumin whereas percentage of cells in S phase was increased. We have also observed that the levels of dead cells determined by DNA fragmentation and membrane permeabilization caused by serum starvation were not significantly altered by the inclusion of albumin. Therefore, the increase in cell number was mainly caused by albumin promoting proliferation rather than protection against cell death. These primary findings demonstrate that albumin has immediate effects on HepG2/C3A hepatocellular carcinoma cells. These effects should be taken into consideration when studying the effects of albumin bound drugs or pathological ligands bound to albumin on HepG2/C3A cells.

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37 ## Abstract

38 Albumin is the most abundant plasma protein and functions as a transport molecule that
39 continuously interacts with various cell types. Because of these properties, albumin has been
40 exploited by the pharmaceutical industry to improve drug delivery to target cells. The immediate
41 effects of albumin on cells however requires further understanding. The cell interacting
42 properties and pharmaceutical applications of albumin incentivises continual research into the
43 immediate effects of albumin on cells. The HepG2/C3A hepatocellular carcinoma cell line is
44 used as a model for studying cancer pathology as well as liver biosynthesis and cellular
45 responses to drugs. Here we investigated the direct effect of purified albumin on HepG2/C3A
46 cell proliferation in the absence of serum, growth factors and other serum originating albumin
47 bound molecules. We demonstrated that serum starvation causes HepG2/C3A cell numbers to
48 stagnate, whereas inclusion of albumin allows an increase in cell numbers. Cell cycle analysis
49 demonstrated that the percentage of cells in G1 phase during serum starvation was reduced by
50 the inclusion of albumin whereas percentage of cells in S phase was increased. We have also
51 observed that the levels of dead cells determined by DNA fragmentation and membrane
52 permeabilization caused by serum starvation were not significantly altered by the inclusion of
53 albumin. Therefore, the increase in cell number was mainly caused by albumin promoting
54 proliferation rather than protection against cell death. These primary findings demonstrate that
55 albumin has immediate effects on HepG2/C3A hepatocellular carcinoma cells. These effects
56 should be taken into consideration when studying the effects of albumin bound drugs or
57 pathological ligands bound to albumin on HepG2/C3A cells.

58

59 ## Introduction

60 Albumin comprises half of the plasma proteins in healthy individuals at concentrations of circa
61 40 g/L (0.6 mmol/L) and is produced by hepatocytes and exported through the blood to the rest
62 of the cells in the body (Margarson & Soni, 1998; Quinlan, Martin & Evans, 2005). Albumin is
63 capable of traversing intracellularly between different organs due to its interactions with several
64 cellular receptors. Hence, many pharmaceutical manufacturers bind drugs to albumin in order to
65 improve their circulatory half-life (Dennis et al., 2002; Sleep, Cameron & Evans, 2013) and
66 delivery into target cells (Frei, 2011; Sleep, 2015; Larsen et al., 2016; Azizi et al., 2017).
67 Albumin's ability to transport between different cell occurs through endocytosis and transcytosis
68 and is controlled by several cellular receptors. These interactions dictate whether albumin should
69 be internalised or cross the vascular endothelial barrier to extravascular compartments (Schnitzer
70 et al., 1992; Schnitzer & Oh, 1994; Vogel et al., 2001). These cellular receptors are selective
71 when binding albumin based on its ligand profile. This can be exemplified in glycoprotein
72 receptors gp60, gp18 and gp30. Native albumin binds to gp60 and is transported by transcytosis
73 through the endothelial cells whereas modified albumin (by surface adsorption to colloidal Au or
74 maleic anhydride treatment) binds gp18 and gp30 and is internalised by endocytosis to be
75 delivered to lysosomes for degradation (Schnitzer et al., 1992; Schnitzer & Bravo, 1993;
76 Schnitzer & Oh, 1994). Another study demonstrates neonatal Fc receptor (FcRn) in transporting

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77 albumin across endothelial cells. Recombinant albumin was engineered to have high or low
78 affinity to bind FcRn. Albumin with high affinity was recycled whereas low affinity albumin
79 underwent lysosomal degradation (Schmidt et al., 2017). These cellular interactions with
80 albumin demonstrate that cells selectively dictate the fate of albumin based on the albumin's
81 ligand conformation. This raises the question of whether the cells in turn respond physiologically
82 to albumin.
83 Several studies have indeed demonstrated that different cell types not only respond to albumin,
84 but they respond differently. One of the prominent effects of albumin on cells can be
85 demonstrated by albumins role in apoptosis. Albumin has shown to protect against apoptosis
86 during serum starvation in several cell types including endothelial cells (Zoellner et al., 1996),
87 Pheochromocytoma cells (Zhang et al., 2012) and Neuroblastoma cells (Gallego-Sandín et al.,
88 2005). Albumin also protects against ROS activated apoptosis in chronic lymphocytic leukaemia
89 cells (Moran et al., 2002) and hybridoma T cells (Liu et al., 2012). Albumin can also have a
90 detrimental role, such as that in proximal tubular cells, by causing endoplasmic reticulum stress
91 that consequently leads to apoptosis (Ohse et al., 2006). For its beneficiary role, albumin is used
92 as a supplement in serum free media (Guilbert & Iscove, 1976; Jäger, Lehmann & Friedl, 1988;
93 Francis, 2010) implying its importance in cell maintenance. These effects of albumin however
94 need further interpretation.
95 It has become evident that the diversity of cellular responses to albumin is not only dependent on
96 the cell type but also on the properties of the interacting albumin. This diversity was
97 demonstrated in rat kidney, human squamous carcinoma and various human neuronal cells
98 having responses to fraction V albumin (HPLC fraction of albumin that contains impurities,
99 mainly fatty acids) that are different from their responses to fatty acid free albumin (Keenan et
100 al., 1997; Hooper, Taylor & Pocock, 2005). Effects of albumin on hepatocellular carcinoma cell
101 line have been previously studied demonstrating conflicting outcomes. While one study
102 suggested that albumin stimulates proliferation of hepatocellular carcinoma cells that were
103 inhibited by fatty acids (Lystad et al., 1994), other studies suggested hepatocellular carcinoma
104 cells cease proliferation in response to albumin (Nojiri & Joh, 2014; Bağırsakçı et al., 2017a).
105 From the collectively reviewed experiments, it can be extrapolated that the diverse cellular
106 responses to albumin are attributed to the varying albumin receptors in the cell as well as the
107 varying ligand profiles of albumin.
108 Although, studies demonstrated diverse responses of cellular interactions with albumin, more
109 research is still needed to understand the specifics of these cellular responses. Here we study the
110 effect of albumin on HepG2/C3A hepatocellular carcinoma cell line, a contact inhibited subclone
111 of the HepG2 cell line (Aden et al., 1979) that retains some physiological functions of normal
112 hepatocytes (Kelly et al., 1992; Nibourg et al., 2012). The HepG2/C3A cells are used as a model
113 cell line for studying parenchymal biosynthesis (Knowles, Howe & Aden, 1980; Zannis et al.,
114 1981; Nibourg et al., 2012) and screening for cytotoxicity of drug compounds for side effects
115 involving liver injury (Gaskell et al., 2016; Doß et al., 2017). The cell line has also been
116 considered for testing clinical samples in screening for diseases (Sauer et al., 2012, 2018).

117 Furthermore, the HepG2/C3A cell line has been used in clinical trials as a therapeutic in an
118 extracorporeal bioartificial liver device (Nibourg et al., 2012).
119 Since hepatocytes play an important role in albumin metabolism and albumin bound drug
120 clearance (Meijer & van der Sluijs, 1989), we decided to evaluate the HepG2/C3A cell line's
121 immediate responses to albumin. A simple approach is required to understand basic responses of
122 HepG2/C3A cells to albumin prior to engaging in complex experiments in order to exploit these
123 features for testing albumin bound drugs or effects of different bound albumin profiles on cells.
124 Therefore, we applied charcoal defatted human serum albumin to cultured HepG2/C3A cells in
125 the absence of serum. The immediate responses of the cells, particularly proliferation and cell
126 death, were examined. Proliferation was evaluated through changes in cell counts and cell cycle
127 analysis whereas cell death was measured by analysing apoptotic DNA fragmentation and
128 membrane permeabilization. Similar studies have been performed on the HepG2 hepatocellular
129 carcinoma parent clone demonstrating that albumin prevents proliferation (Nojiri & Joh, 2014;
130 Bağırsakçı et al., 2017a). In this study, the experiments were carried out on the HepG2/C3A
131 subclone and were contradictory to these earlier findings. Our results contrarily suggest that
132 albumin promotes proliferation of serum starved HEPG2/C3A hepatocellular carcinoma cells by
133 allowing G1 to S phase cell cycle transition. We also show that albumin does not significantly
134 interfere in cell death by apoptosis or necrosis. This primary approach offers a platform and a
135 control method when studying the effects of drug bound albumin and pathologically modified
136 albumin on cell proliferation as well as cytotoxicity.
137

138 Materials & Methods

139 Cell culture

140 The cell line used in this study is C3A [HepG2/C3A, derivative of Hep G2 (ATCC HB-8065)]
141 (ATCC® CRL-10741™). Cells were routinely cultured in DMEM (Gibco) supplemented with
142 10% foetal bovine serum (FBS, Sigma) and 2mM L-glutamine (Biochrom) at a seeding density
143 of 8×10^{4} cells/cm^2 in 25 cm^2 flasks (Greiner Bio-one) (supplementary fig S1). Cells were
144 incubated at 37°C, 98% humidity and 5% CO_2 (BBD 6220 CO_2 incubator, Thermo Scientific).
145 Cells were harvested by detachment using trypsin/EDTA, then neutralised with media containing
146 10% FBS. They were then pelleted by centrifugation at 300 x g, resuspended in media composed
147 of the desired treatments at a total of 2×10^{6} cells (8×10^{4} cells/cm^2). Cell counts were carried
148 out under a light microscope using a Neubauer haemocytometer.
149 The treatments in this study include serum free media (DMEM with 2mM L-glutamine,
150 completely without FBS) as the serum starved control, serum free media containing 5mg/ml
151 albumin (human serum albumin, Octapharma) or serum free media containing 5mg/ml dextran
152 70 (Carl Roth). The albumin used in this study is in physiological solution and charcoal treated
153 (Hepalbin, Albutec) to reduce albumin bound stabilisers and other fatty acids prior to applying it
154 to the cells (Chen, 1967). A concentration of 5 mg/ml albumin was applied in our experiments
155 because it is comparable to total protein concentrations in 10% FBS used in routine cell culture.

156 HepG2 cells typically respond slowly to serum starvation and evidence of cell cycle arrest and
157 apoptosis are usually delayed (Fig. S2 and Fig. S3) (Zhuge & Cederbaum, 2006), therefore tests
158 were conducted after 72 hours in culture. Cells were routinely viewed using inverted phase
159 contrast microscope (DM IL LED, Leica) and images were captured using a mounted camera
160 (MC120 HD, Leica).
161 HepG2/C3A cells were authenticated using short tandem repeat analysis and hepatocyte
162 functionality was confirmed by measuring micro albumin synthesis and cytochrome P450
163 monooxygenase 1A activity. Cells were periodically screened for mycoplasma by extranuclear
164 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Carl Roth) staining viewed under
165 ECLIPSE Ti inverted fluorescence microscope (Nikon). Confirmatory testing of mycoplasma
166 enzymatic activity (Mycoalert, Lonza) was carried out according to kit instructions and measured
167 using CLARIOstar plate reader (BMG LABTECH). Both tests have been negative confirming
168 absence of mycoplasma contamination.

169 **Cell cycle analysis**

170 Harvested cells were fixed in 66% Ethanol at a concentration of 10^6 cells/ml for a minimum of 2
171 hours at 4° C. Cells were washed with phosphate buffer saline and incubated with 200µl staining
172 solution consisting of 50 µg/ml propidium iodide and 550 U/ml RNase A (Abcam) in the dark at
173 37° C for 20 to 30 minutes. Measurements were taken at excitation wave length of 488nm using
174 the FACSVerse flow cytometer (BD Biosciences). Gating was carried out using FlowJo 10.5.3
175 software (FlowJo) and calculated using the software's Watson Pragmatic algorithm to correct for
176 overlaps between the peaks.

177 **TUNEL assay**

178 Suspended cells were fixed with 1% paraformaldehyde (pH 7.4) for 60 minutes on ice. The cells
179 were washed with PBS followed by 70% ethanol fixation at -20° C overnight. Cells were treated
180 with terminal deoxynucleotidyl transferase enzyme (TdT) and fluorescein isothiocyanate (FITC)
181 deoxy uridine triphosphate (FITC-dUTP) provided in the kit (Phoenix flow systems).
182 Measurements were taken at excitation wave length of 488nm using the FACSVerse flow
183 cytometer (BD Biosciences).

184 **Fluorescence imaging**

185 Cells were cultured on a 24 well plate (Greiner) at a seeding density of 8 x 10^4 cells/cm^2 for 72
186 hours with the different treatments. Unfixed adherent cells were directly stained with a mixture
187 of 0.6 µM calcein AM (Invitrogen), 2 µM ethidium bromide (Invitrogen) and 4 µM 4',6-
188 Diamidine-2'-phenylindole dihydrochloride (DAPI, Carl Roth) and incubated at 37°C for 20
189 minutes. The cells were viewed and photographed under ECLIPSE Ti inverted fluorescence
190 microscope (Nikon). Cells positive for membrane permeabilization were measured as the
191 proportion of cells stained with ethidium bromide from total cell numbers stained with DAPI. A
192 minimum of 1000 cells and three fields of view were counted per well.

193 **Statistics**

194 Four biological replicates were used in each treatment. F test was used to determine equal
195 variances. Two tailed T-test assuming equal or unequal (Welch test) variances was carried out

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196 accordingly to determine statistical significance when comparing two groups. One way-ANOVA
197 followed by Bonferroni's post hoc analysis was carried out when comparing multiple groups
198 against the control. P value of less than 0.05 is considered significant (α=0.05).
199

200 Results

201 Albumin alters cell morphology and results in increased cell counts in serum 202 starved HEPG2/C3A cells

203 To test the immediate effects of albumin, cells were cultured in serum free media containing
204 5mg/ml albumin and compared with the cells cultured in serum free media without albumin
205 (serum starved control). Cells cultured in serum free media containing 5 mg/ml dextran, with a
206 comparable molecular weight to albumin (70 kDa), was carried out as a control for effects that
207 might relate to oncotic pressure.
208 Serum starved controls displayed a slight irregular morphology but retained the epithelial
209 polygonal shape and remained as monolayers in colonies (Fig. 1a). Noticeable morphological
210 differences were observed in albumin treated cells when compared to the serum starved control.
211 Cells remained in colonies but displayed a rounded morphology, formed clusters and grew in
212 more than one layer (Fig. 1b). Dextran treated cells demonstrated a morphological effect more in
213 resemblance to serum starved cells (Fig. 1c) and did not exhibit the rounded morphology,
214 clusters and layering seen in albumin treated cells.
215 The effects of albumin on cell counts of serum starved cells was evaluated in comparison to
216 Dextran treated cells and serum starved control. Data passed equal variance test and were
217 analysed by one-way ANOVA followed by Bonferroni's multiple comparison versus control
218 (serum starved cells). Serum starvation for 72 hours yielded a mean cell count of $2.8x10^6$ cells.
219 Inclusion of albumin in serum starved cells cultured for 72 hours significantly increased mean
220 cell counts to $4x10^6$ cells (p=0.019, n=4, α=0.05). Dextran treatment of serum starved cells had
221 fewer cells ($2x10^6$ cells) than the serum starved control but the difference was not significant
222 (p=0.14, n=4, α=0.05) (Fig. 1d). These findings suggest that albumin alters cell morphology and
223 results in increased cell counts in serum starved cells. These effects are not simply due to
224 osmotic pressure and are specific for albumin as these results could not be reproduced in cultures
225 treated with an equal molecular weight of Dextran.

226 Albumin promotes proliferation but does not prevent cell death in serum starved 227 HEPG2/C3A cells

228 Albumin triggered increased cell counts in serum starved HEPG2/C3A cells requires further
229 explanation into whether these effects are a result of promoting proliferation or preventing cell
230 loss. HEPG2/C3A cells cultured in media containing 10% FBS were maintained for 120 hours
231 until confluent (Fig. S1b) and possibly contact inhibited (Kelly et al., 1992; Davis, Ho & Dowdy,
232 2001; Cho et al., 2005). Cell cycle analysis demonstrated that the cells cultured in 10% FBS for
233 120 hours were 86.6 % in G1 phase, 6.2 % in S phase and 7.2 % in G2/M phase (Fig. S1c). The
234 confluent cells were harvested then seeded at the regular seeding density ($2x10^6$ cells) in serum
235 starved media and serum starved media containing 5mg/ml albumin. Cell cycle analysis after 72

236 hours in culture demonstrated a significant reduction in G1 phase in albumin treated cells
237 (78.3±3.2 %) compared to serum starved cells (86.4±2.3 %) (equal variance t.test, p=0.006, n=4,
238 α=0.05). Whereas S phase was significantly higher in albumin treated cells (14.3±3.6 %)
239 compared to serum starved cells (6.5±1.5 %) (unequal variance (Welch) t.test, p=0.016, n=4,
240 α=0.05). There was however no significant difference in G2/M phase between albumin treated
241 cells (7.4±1.9 %) and serum starved cells (7±2.2 %) (equal variance t.test, p=0.8, n=4, α=0.05)
242 (Fig. 2 and Fig. S2). These results suggest that serum starvation resulted in a cell cycle arrest in
243 G1 phase, while inclusion of albumin promoted cell cycle transition into S phase.
244 Serum starvation causes cell apoptosis and necrosis in cultured cells. The effects of serum
245 starvation are however slow in HepG2 cells (Zhuge & Cederbaum, 2006; Liang et al., 2013).
246 This slow response is recapitulated in HEPG2/C3A cells (Fig. S3 and Fig. S4); hence, the 72-
247 hour time point was selected for this study. Apoptosis and necrosis were measured in
248 HEPG2/C3A cells by TUNEL assay and ethidium bromide staining respectively. Serum starved
249 cells were 16.6 ± 7.2 % apoptotic and albumin treated cells were 11.6 ± 10.2 % apoptotic (Fig.
250 3). Although TUNEL positive cells were perceptibly lower in some of the samples when treated
251 with albumin (Fig. S5), the effect was not statistically significant (unequal variance (Welch)
252 t.test, n=4, p=0.46, α=0.05) (Fig. 3c).
253 Cells in culture for 72 hours were stained with calcein AM (green) and ethidium bromide (red) to
254 demonstrate presence of live and necrotic cells respectively (Decherchi, Cochard & Gauthier,
255 1997). DAPI (blue) nuclear stain was carried out to quantify total number of cells in the field of
256 view. Live cells were characterised by esterase activity that causes calcein AM to fluoresce in the
257 cytoplasm and is clearly present in all treatments. Necrotic cells were characterised by damaged
258 cell membrane that is permeable to ethidium bromide allowing it to stain the nucleus. Serum
259 starvation resulted in 13.8 ± 4.8 % necrosis. Inclusion of 5mg/ml albumin resulted in an increase
260 in necrosis to 16.9 ± 8.9 % but is not significantly higher than serum starved cultures (equal
261 variance t.test, n=4, p=0.5, α=0.05). Cells grown in 10% FBS were used as negative controls for
262 TUNEL assay (0.8 ± 0.2 % apoptotic) and ethidium bromide staining (1.7 ± 0.8% necrotic) (Fig.
263 S6). While inclusion of albumin significantly accounts for an increase in cell counts and
264 proportion of cells in S phase during serum starvation it does not significantly protect against cell
265 death by either apoptosis or necrosis.

266 **Discussion and conclusions**

267 The frequent interactions of naturally abundant albumin with cells in the body as well as its wide
268 exogenous applications in the biomedical and pharmaceutical industries (Dennis et al., 2002;
269 Sleep, Cameron & Evans, 2013) prompted us to study the effects of albumin on cells. We have
270 selected HepG2/C3A carcinoma cells because they originate from hepatocytes (Aden et al.,
271 1979) and have been used as a model for studying hepatocellular carcinoma (Chen et al., 2015;
272 Ao et al., 2017) as well as normal hepatocyte functions (Nibourg et al., 2012; Gaskell et al.,
273 2016). Hepatocytes play an important role in the clearance of albumin bound substances (Meijer
274 & van der Sluijs, 1989), therefore some of these albumin interacting properties can potentially be
275 used in targeting hepatocellular carcinoma or studying the effects of pathologically modified

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276 albumin on cellular function. This study aims to determine immediate effects of albumin on
277 HepG2/C3A cell proliferation and death during serum starvation.
278 Although, the effects of albumin on the cell cycle of hepatocellular carcinoma cells have been
279 previously studied, the outcomes of those studies are debatable. They argued that albumin
280 inhibits tumour evidenced by its increased levels correlating with cancer remission and addition
281 of albumin to serum starved hepatoma cells caused G1 arrest (Nojiri & Joh, 2014; Bağırsakçı et
282 al., 2017b). It is worth noting that serum is essential for hepatocellular carcinoma growth (Zhuge
283 & Cederbaum, 2006; Liang et al., 2013) whereas serum starvation is routinely carried out to
284 arrest cells in G1 phase (Davis, Ho & Dowdy, 2001; Langan, Rodgers & Chou, 2017). The
285 observations that albumin causes cell cycle arrest might have been an artefact.
286 We carried out our experiments on HEPG2/C3A cells, a selected colony of HepG2 cells, that are
287 responsive to contact inhibition. In our approach, the cells were collected for experimentation
288 after they were grown to confluency to synchronize them by means of contact inhibition (Kelly
289 et al., 1992; Davis, Ho & Dowdy, 2001; Cho et al., 2005). Cells were then grown under serum
290 starved conditions with or without albumin. The cells were counted and analysed for cell cycle
291 after 72-hour treatments. We observed an increase in cell counts that correlated with a reduction
292 in percentages of cells in G1 phase and an increase in S phase suggestive of cell cycle
293 progression. To further rule out proliferation inhibitory effects of albumin that were suggested in
294 the studies mentioned above (Nojiri & Joh, 2014; Bağırsakçı et al., 2017b), we treated
295 HEPG2/C3A cells grown in media containing FBS with 10mg/ml albumin and did not observe a
296 significant change in cell counts (equal variance t.test, n=3, p=0.36) (Fig. S7).
297 The initial observed effects of albumin on serum starved HEPG2/C3A cells were morphological.
298 Serum starved cells retain epithelial morphology and ability to spread. Albumin caused cells to
299 appear more round, small and clustered (Fig. 1). Addition of albumin had also significantly
300 increased cell counts in serum starved cells. These effects altogether suggest that albumin plays
301 an immediate role in cellular physiology. We proceeded to investigate other cell dynamics to
302 determine whether the increase in cell counts by addition of albumin is primarily due to
303 promotion of cell proliferation or protection of cells from death.
304 During serum starvation, earlier time points did not demonstrate sufficient cell cycle arrest
305 whereas at later time points most cells were dead. A time point of 72 hours after serum starvation
306 was selected due to minimal cell loss (mean number of cells harvested were not lower than cells
307 seeded) and sufficient G1 cell cycle arrest was observed (Fig. S3). Addition of albumin to the
308 serum starved cells increased cell counts, resulted in a decrease in G1 phase and an increase in S
309 phase. This suggests that albumin promotes cell proliferation by permitting cell cycle transition
310 from G1 to S phase.
311 Cell death was measured to rule out a protective effect of albumin. TUNEL assay was carried out
312 to determine cells that underwent DNA fragmentation, an indicator of apoptosis (Crowley,
313 Marfell & Waterhouse, 2016). Nuclear staining of cells with ethidium bromide was carried out to
314 demonstrate membrane damage as a result of necrosis or secondary necrosis (Zong & Thompson,
315 2006). Inclusion of albumin in serum starved cells did not significantly change the proportion of

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316 dead cells identified by these methods. This suggest that albumin does not protect against DNA
317 fragmentation and membrane permeabilization that occurs during serum starvation.
318 We have argued that albumin has an immediate effect on cell proliferation by promoting cell
319 cycle transition from G1 to S phase in the absence of serum and other proteins including growth
320 promoting factors. However, we did not study whether albumin directly interfered with
321 molecular pathways of transcription factors involved in cell cycle transition or indirectly
322 influenced proliferation through interfering with other processes such as cell spreading.
323 We investigated modes of cell death individually, but we did not examine any overlaps between
324 DNA fragmentation and membrane permeabilization. Furthermore, we did not examine whether
325 the underlying cause of death in the membrane permeabilized cells was apoptosis or direct
326 necrosis. The tests simply suggest that albumin did not prevent various fates of cell death that
327 occur during serum starvation.
328 These findings argue that albumin resulted in increased cell counts as a result of increased
329 proliferation through promotion of G1 to S phase transition and not by prevention of cell death in
330 serum starved HepG2/C3A cells. This study offers primary results and a platform for further
331 investigations into the molecular interactions of albumin with cells. Additionally, this approach
332 can be used as a control to study different modifications of albumin, ligand profiles and drug
333 bound albumin.
334

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341

342 ## References

343 Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. 1979. Controlled synthesis of HBsAg
344 in a differentiated human liver carcinoma-derived cell line. *Nature* 282:615–6. DOI:
345 10.1038/282615a0.
346 Ao L, Guo Y, Song X, Guan Q, Zheng W, Zhang J, Huang H, Zou Y, Guo Z, Wang X. 2017.
347 Evaluating hepatocellular carcinoma cell lines for tumour samples using within-sample relative
348 expression orderings of genes. *Liver International* 37:1688–1696. DOI: 10.1111/liv.13467.
349 Azizi M, Ghourchian H, Yazdian F, Dashtestani F, AlizadehZeinabad H. 2017. Cytotoxic effect
350 of albumin coated copper nanoparticle on human breast cancer cells of MDA-MB 231. *PLOS*
351 *ONE* 12. DOI: 10.1371/journal.pone.0188639.
352 Bağırsakçı E, Şahin E, Atabey N, Erdal E, Guerra V, Carr BI. 2017a. Role of Albumin in Growth
353 Inhibition in Hepatocellular Carcinoma. *Oncology* 93:136–142. DOI: 10.1159/000471807.

354 Bağırsakçı E, Şahin E, Atabey N, Erdal E, Guerra V, Carr BI. 2017b. Role of Albumin in
355 Growth Inhibition in Hepatocellular Carcinoma. *Oncology* 93:136–142. DOI:
356 10.1159/000471807.
357 Chen RF. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *The Journal*
358 *of biological chemistry* 242:173–81.
359 Chen B, Sirota M, Fan-Minogue H, Hadley D, Butte AJ. 2015. Relating hepatocellular
360 carcinoma tumor samples and cell lines using gene expression data in translational research.
361 *BMC Medical Genomics* 8:S5. DOI: 10.1186/1755-8794-8-S2-S5.
362 Cho S, Ock S, Yoo J, Mohana kumar B, Choe S, Rho G. 2005. Effects of Confluent, Roscovitine
363 Treatment and Serum Starvation on the Cell-cycle Synchronization of Bovine Foetal Fibroblasts.
364 *Reproduction in Domestic Animals* 40:171–176. DOI: 10.1111/j.1439-0531.2005.00577.x.
365 Crowley LC, Marfell BJ, Waterhouse NJ. 2016. Detection of DNA Fragmentation in Apoptotic
366 Cells by TUNEL. *Cold Spring Harbor Protocols* 2016. DOI: 10.1101/pdb.prot087221.
367 Davis PK, Ho A, Dowdy SF. 2001. Biological Methods for Cell-Cycle Synchronization of
368 Mammalian Cells. *BioTechniques* 30:1322–1331. DOI: 10.2144/01306rv01.
369 Decherchi P, Cochard P, Gauthier P. 1997. Dual staining assessment of Schwann cell viability
370 within whole peripheral nerves using calcein-AM and ethidium homodimer. *Journal of*
371 *neuroscience methods* 71:205–13. DOI: 10.1016/S0165-0270(96)00146-X.
372 Dennis MS, Zhang M, Meng YG, Kadkhodayan M, Kirchhofer D, Combs D, Damico LA. 2002.
373 Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *The*
374 *Journal of biological chemistry* 277:35035–43. DOI: 10.1074/jbc.M205854200.
375 Doß S, Potschka H, Doß F, Mitzner S, Sauer M. 2017. Hepatotoxicity of Antimycotics Used for
376 Invasive Fungal Infections: In Vitro Results. *BioMed Research International* 2017:9658018.
377 DOI: 10.1155/2017/9658018.
378 Francis GL. 2010. Albumin and mammalian cell culture: implications for biotechnology
379 applications. *Cytotechnology* 62:1–16. DOI: 10.1007/s10616-010-9263-3.
380 Frei E. 2011. Albumin binding ligands and albumin conjugate uptake by cancer cells.
381 *Diabetology & metabolic syndrome* 3. DOI: 10.1186/1758-5996-3-11.
382 Gallego-Sandín S, Novalbos J, Rosado A, Cano-Abad MF, Arias E, Abad-Santos F, García AG.
383 2005. Albumin prevents mitochondrial depolarization and apoptosis elicited by endoplasmic
384 reticulum calcium depletion of neuroblastoma cells. *European Journal of Pharmacology* 520:1–
385 11. DOI: 10.1016/j.ejphar.2005.06.044.
386 Gaskell H, Sharma P, Colley HE, Murdoch C, Williams DP, Webb SD. 2016. Characterization
387 of a functional C3A liver spheroid model. *Toxicology Research* 5:1053–1065. DOI:
388 10.1039/C6TX00101G.
389 Guilbert LJ, Iscove NN. 1976. Partial replacement of serum by selenite, transferrin, albumin and
390 lecithin in haemopoietic cell cultures. *Nature* 263:594–5. DOI: 10.1038/263594a0.
391 Hooper C, Taylor DL, Pocock JM. 2005. Pure albumin is a potent trigger of calcium signalling
392 and proliferation in microglia but not macrophages or astrocytes. *Journal of Neurochemistry*
393 92:1363–1376. DOI: 10.1111/j.1471-4159.2005.02982.x.

394 Jäger V, Lehmann J, Friedl P. 1988. Serum-free growth medium for the cultivation of a wide
395 spectrum of mammalian cells in stirred bioreactors. *Cytotechnology* 1:319–29. DOI:
396 10.1007/BF00365077.
397 Keenan J, Dooley M, Pearson D, Clynes M. 1997. Recombinant Human Albumin in Cell
398 Culture: Evaluation of Growth-Promoting Potential for NRK and SCC-9 Cells In Vitro.
399 *Cytotechnology* 24:243–52. DOI: 10.1023/A:1007916930200.
400 Kelly JH, Koussayer T, He D, Chong MG, Shang TA, Whisennand HH, Sussman NL. 1992.
401 Assessment of an extracorporeal liver assist device in anhepatic dogs. *Artificial organs* 16:418–
402 22. DOI: 10.1111/j.1525-1594.1992.tb00543.x.
403 Knowles BB, Howe CC, Aden DP. 1980. Human hepatocellular carcinoma cell lines secrete the
404 major plasma proteins and hepatitis B surface antigen. *Science (New York, N.Y.)* 209:497–9.
405 DOI: 10.1126/science.6248960.
406 Langan TJ, Rodgers KR, Chou RC. 2017. Synchronization of Mammalian Cell Cultures by
407 Serum Deprivation. In: *Methods in molecular biology (Clifton, N.J.)*. 97–105. DOI: 10.1007/978-
408 1-4939-6603-5_6.
409 Larsen MT, Kuhlmann M, Hvam ML, Howard KA. 2016. Albumin-based drug delivery:
410 harnessing nature to cure disease. *Molecular and Cellular Therapies* 4:3. DOI: 10.1186/s40591-
411 016-0048-8.
412 Liang C, Chen W, Zhi X, Ma T, Xia X, Liu H, Zhang Q, Hu Q, Zhang Y, Bai X, Liang T. 2013.
413 Serotonin promotes the proliferation of serum-deprived hepatocellular carcinoma cells via
414 upregulation of FOXO3a. *Molecular cancer* 12:14. DOI: 10.1186/1476-4598-12-14.
415 Liu S-Y, Chen C-L, Yang T-T, Huang W-C, Hsieh C-Y, Shen W-J, Tsai T-T, Shieh C-C, Lin C-
416 F. 2012. Albumin prevents reactive oxygen species-induced mitochondrial damage, autophagy,
417 and apoptosis during serum starvation. *Apoptosis* 17:1156–1169. DOI: 10.1007/s10495-012-
418 0758-6.
419 Lystad E, Høstmark AT, Kiserud C, Haugen A. 1994. Influence of fatty acids and bovine serum
420 albumin on the growth of human hepatoma and immortalized human kidney epithelial cells. *In
421 vitro cellular & developmental biology. Animal* 30A:568–73. DOI: 10.1007/BF02631254.
422 Margarson MP, Soni N. 1998. Serum albumin: touchstone or totem? *Anaesthesia* 53:789–803.
423 DOI: 10.1046/j.1365-2044.1998.00438.x.
424 Meijer DK, van der Sluijs P. 1989. Covalent and noncovalent protein binding of drugs:
425 implications for hepatic clearance, storage, and cell-specific drug delivery. *Pharmaceutical
426 research* 6:105–18. DOI: 10.1023/A:1015961424122.
427 Moran EC, Kamiguti AS, Cawley JC, Pettitt AR. 2002. Cytoprotective antioxidant activity of
428 serum albumin and autocrine catalase in chronic lymphocytic leukaemia. *British journal of
429 haematology* 116:316–28. DOI: 10.1046/j.1365-2141.2002.03280.x.
430 Nibourg GAA, Chamuleau RAFM, van Gulik TM, Hoekstra R. 2012. Proliferative human cell
431 sources applied as biocomponent in bioartificial livers: a review. *Expert opinion on biological
432 therapy* 12:905–21. DOI: 10.1517/14712598.2012.685714.

433 Nojiri S, Joh T. 2014. Albumin suppresses human hepatocellular carcinoma proliferation and the
434 cell cycle. *International journal of molecular sciences* 15:5163–74. DOI:
435 10.3390/ijms15035163.
436 Ohse T, Inagi R, Tanaka T, Ota T, Miyata T, Kojima I, Ingelfinger JR, Ogawa S, Fujita T,
437 Nangaku M. 2006. Albumin induces endoplasmic reticulum stress and apoptosis in renal
438 proximal tubular cells. *Kidney International* 70:1447–1455. DOI: 10.1038/sj.ki.5001704.
439 Quinlan GJ, Martin GS, Evans TW. 2005. Albumin: Biochemical properties and therapeutic
440 potential. *Hepatology* 41:1211–1219. DOI: 10.1002/hep.20720.
441 Sauer M, Haubner C, Mencke T, Nöldge-Schomburg G, Mitzner S, Altrichter J, Stange J. 2012.
442 Impaired cell functions of hepatocytes incubated with plasma of septic patients. *Inflammation*
443 *Research* 61:609–616. DOI: 10.1007/s00011-012-0451-9.
444 Sauer M, Haubner C, Richter G, Ehler J, Mencke T, Mitzner S, Margraf S, Altrichter J, Doß S,
445 Nöldge-Schomburg G. 2018. Impaired Cell Viability and Functionality of Hepatocytes After
446 Incubation With Septic Plasma—Results of a Second Prospective Biosensor Study. *Frontiers in*
447 *Immunology* 9:1448. DOI: 10.3389/fimmu.2018.01448.
448 Schmidt EGW, Hvam ML, Antunes F, Cameron J, Viuff D, Andersen B, Kristensen NN,
449 Howard KA. 2017. Direct demonstration of a neonatal Fc receptor (FcRn)-driven endosomal
450 sorting pathway for cellular recycling of albumin. *The Journal of biological chemistry*
451 292:13312–13322. DOI: 10.1074/jbc.M117.794248.
452 Schnitzer JE, Bravo J. 1993. High affinity binding, endocytosis, and degradation of
453 conformationally modified albumins. Potential role of gp30 and gp18 as novel scavenger
454 receptors. *The Journal of biological chemistry* 268:7562–70.
455 Schnitzer JE, Oh P. 1994. Albondin-mediated capillary permeability to albumin. Differential role
456 of receptors in endothelial transcytosis and endocytosis of native and modified albumins. *The*
457 *Journal of biological chemistry* 269:6072–82.
458 Schnitzer JE, Sung A, Horvat R, Bravo J. 1992. Preferential interaction of albumin-binding
459 proteins, gp30 and gp18, with conformationally modified albumins. Presence in many cells and
460 tissues with a possible role in catabolism. *The Journal of biological chemistry* 267:24544–53.
461 Sleep D. 2015. Albumin and its application in drug delivery. *Expert Opinion on Drug Delivery*
462 12:793–812. DOI: 10.1517/17425247.2015.993313.
463 Sleep D, Cameron J, Evans LR. 2013. Albumin as a versatile platform for drug half-life
464 extension. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1830:5526–5534. DOI:
465 10.1016/j.bbagen.2013.04.023.
466 Vogel SM, Minshall RD, Pilipović M, Tiruppathi C, Malik AB. 2001. Albumin uptake and
467 transcytosis in endothelial cells in vivo induced by albumin-binding protein. *American Journal*
468 *of Physiology-Lung Cellular and Molecular Physiology* 281:L1512–L1522. DOI:
469 10.1152/ajplung.2001.281.6.L1512.
470 Zannis VI, Breslow JL, SanGiacomo TR, Aden DP, Knowles BB. 1981. Characterization of the
471 major apolipoproteins secreted by two human hepatoma cell lines. *Biochemistry* 20:7089–96.
472 DOI: 10.1021/bi00528a006.

473 Zhang L-J, Xue Y-Q, Yang C, Yang W-H, Chen L, Zhang Q-J, Qu T-Y, Huang S, Zhao L-R,
474 Wang X-M, Duan W-M. 2012. Human Albumin Prevents 6-Hydroxydopamine-Induced Loss of
475 Tyrosine Hydroxylase in In Vitro and In Vivo. *PLoS ONE* 7:e41226. DOI:
476 10.1371/journal.pone.0041226.
477 Zhuge J, Cederbaum AI. 2006. Serum deprivation-induced HepG2 cell death is potentiated by
478 CYP2E1. *Free Radical Biology and Medicine* 40:63–74. DOI:
479 10.1016/j.freeradbiomed.2005.08.012.
480 Zoellner H, Höfler M, Beckmann R, Hufnagl P, Vanyek E, Bielek E, Wojta J, Fabry A, Lockie
481 S, Binder BR. 1996. Serum albumin is a specific inhibitor of apoptosis in human endothelial
482 cells. *Journal of cell science* 109 (Pt 10):2571–80.
483 Zong W-X, Thompson CB. 2006. Necrotic death as a cell fate. *Genes & Development* 20:1–15.
484 DOI: 10.1101/gad.1376506.
485

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Figure 1

Comparison of HepG2/C3A cell morphology and cell counts after 72 hour treatments.

Micrographs (20 X objective lens) of cells cultured for 72 hours in (A) serum free media (serum starved control), (B) serum free media containing 5mg/ml albumin and (C) serum free media containing 5mg/ml dextran. Scale bar = 100 µm. (D) Total cell counts after 72 hours in culture. Values are mean ± SD (n=4). *$p < 0.05$, NS= not significant.

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A

B

C

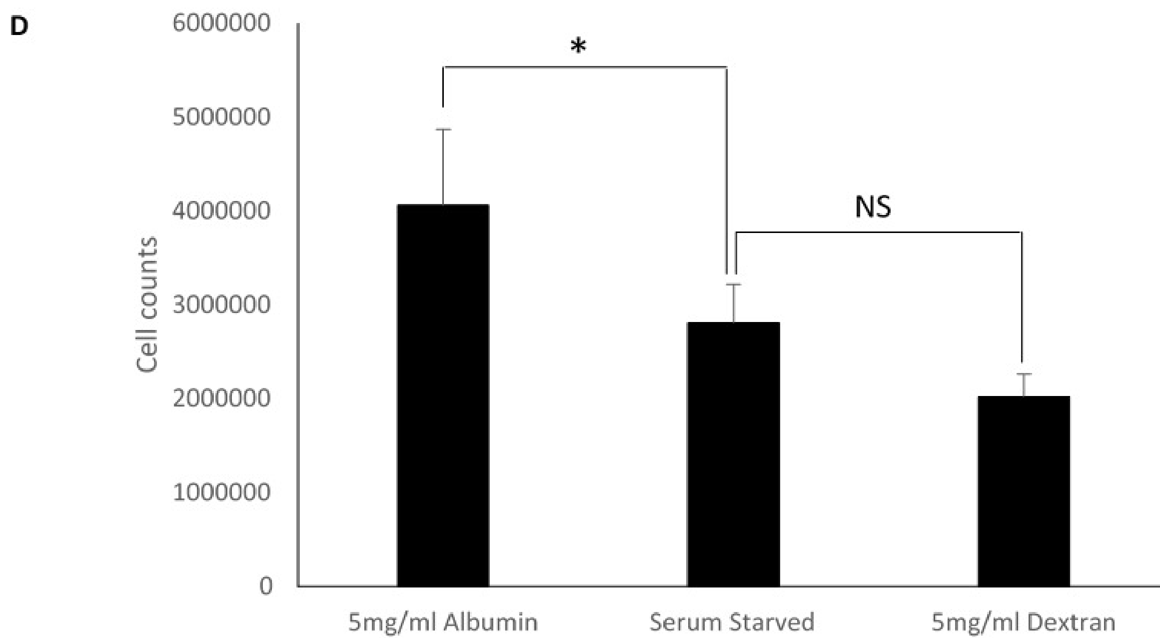

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Figure 2

Cell cycle analysis of serum starved cells with or without albumin.

Flow cytometry histograms of propidium iodide stained cells treated for 72 hours with (A) serum starved media or (B) serum starved media containing 5mg/ml albumin. (C) Bar chart demonstrating the percentage of cell cycle stages calculated from the histogram using the Watson pragmatic algorithm. Values are mean ± SD (n=4). *$p < 0.05$, **$p<0.01$, NS= not significant.

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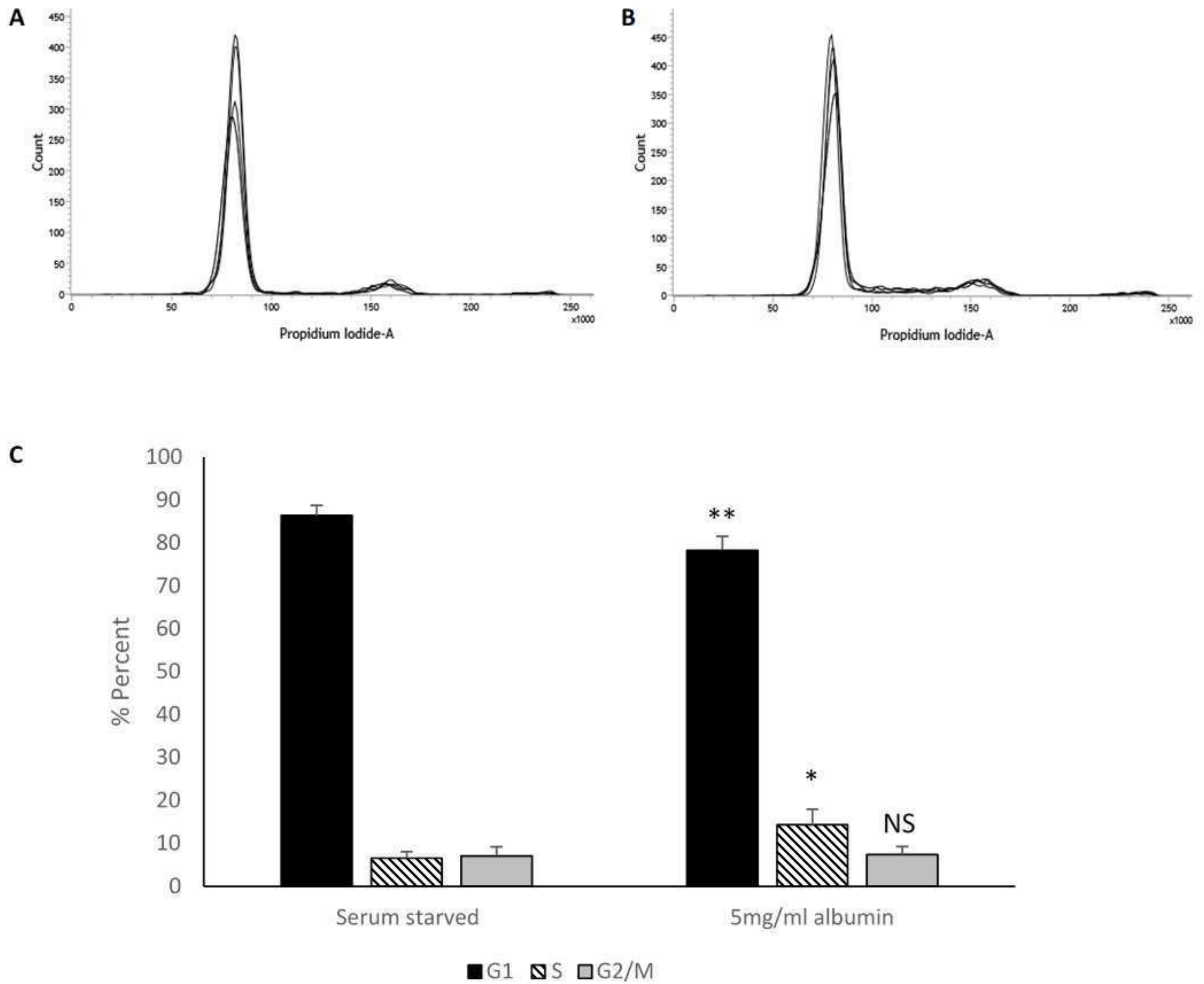
A
Count
Propidium Iodide-A
x1000
B
Count
Propidium Iodide-A
x1000
C
% Percent
Serum starved
5mg/ml albumin
**
*
NS
G1
S
G2/M

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Figure 3

TUNEL assay for the measurement of percentage of cells with fragmented DNA.

Flowcytometry overlay of dot plots of TUNEL positive cells (red) and negative cells (black) cultured for 72 hours in (A) serum starved media or (B) serum starved media containing 5mg/ml albumin. (C) Bar chart demonstrating percentage of TUNEL positive cells. Values are mean ± SD (n=4). NS= not significant.

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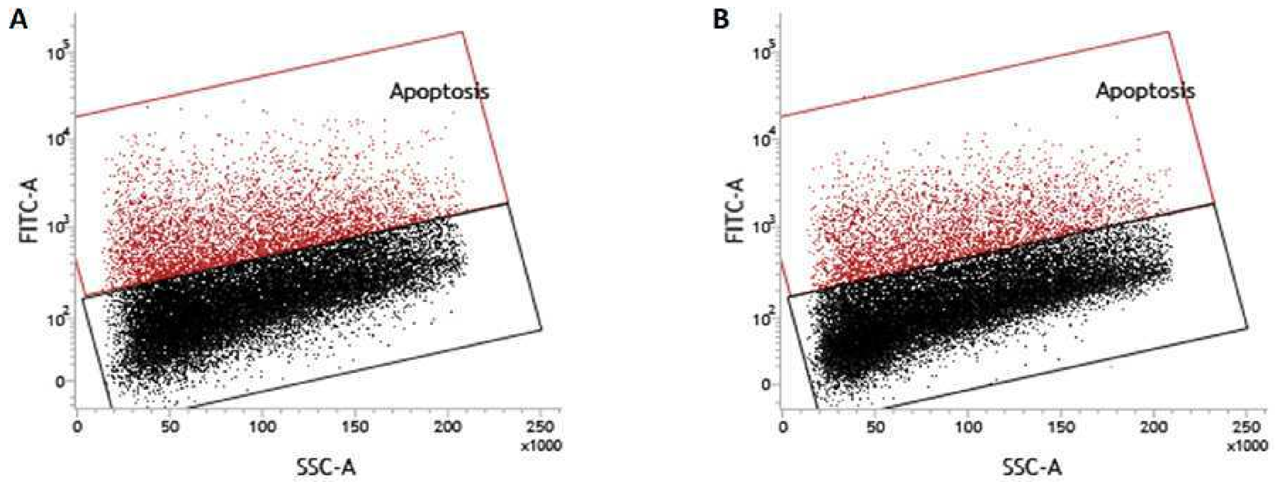
A
B
Apoptosis
Apoptosis
FITC-A
FITC-A
SSC-A
SSC-A
x1000
x1000

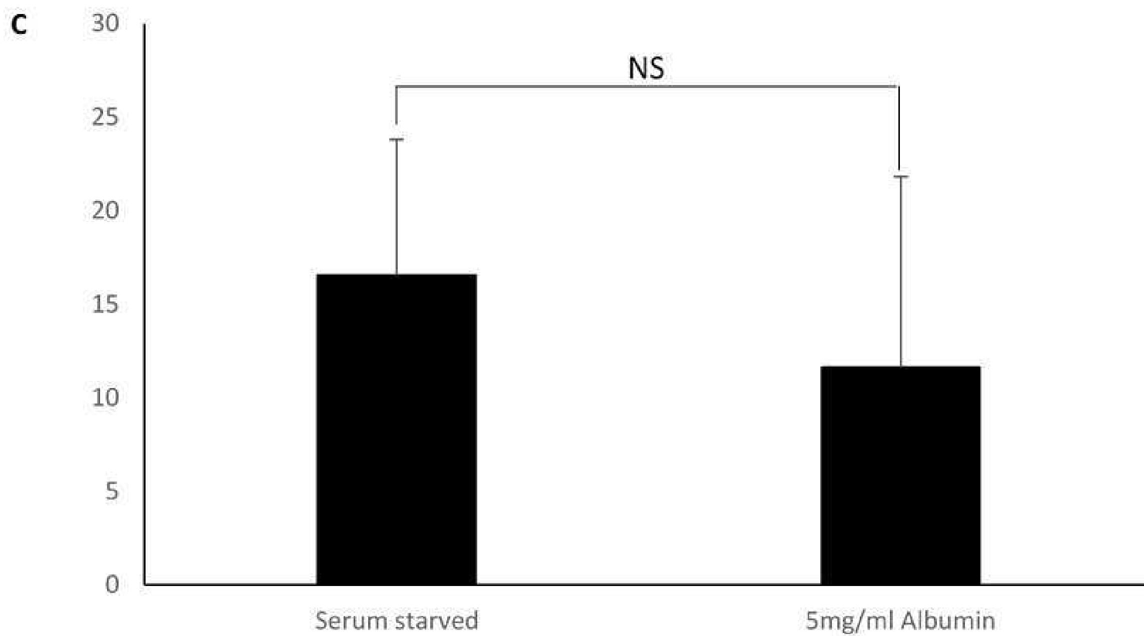
C
NS
Serum starved
5mg/ml Albumin

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Figure 4

Live dead fluorescent micrographs of cells.

Fluorescence microscopy image of cells (10x objective lens) cultured for 72 hours in (A) serum starved media and (B) serum starved media containing 5mg/ml albumin. Fluorochromes used were DAPI (nuclear), calcein AM (cytoplasmic) and ethidium bromide (nuclear). Scale bar = 100 µm. (C) Bar chart demonstrating the differences in necrotic index between the treatments. Values are mean ± SD (n=4). NS= not significant.

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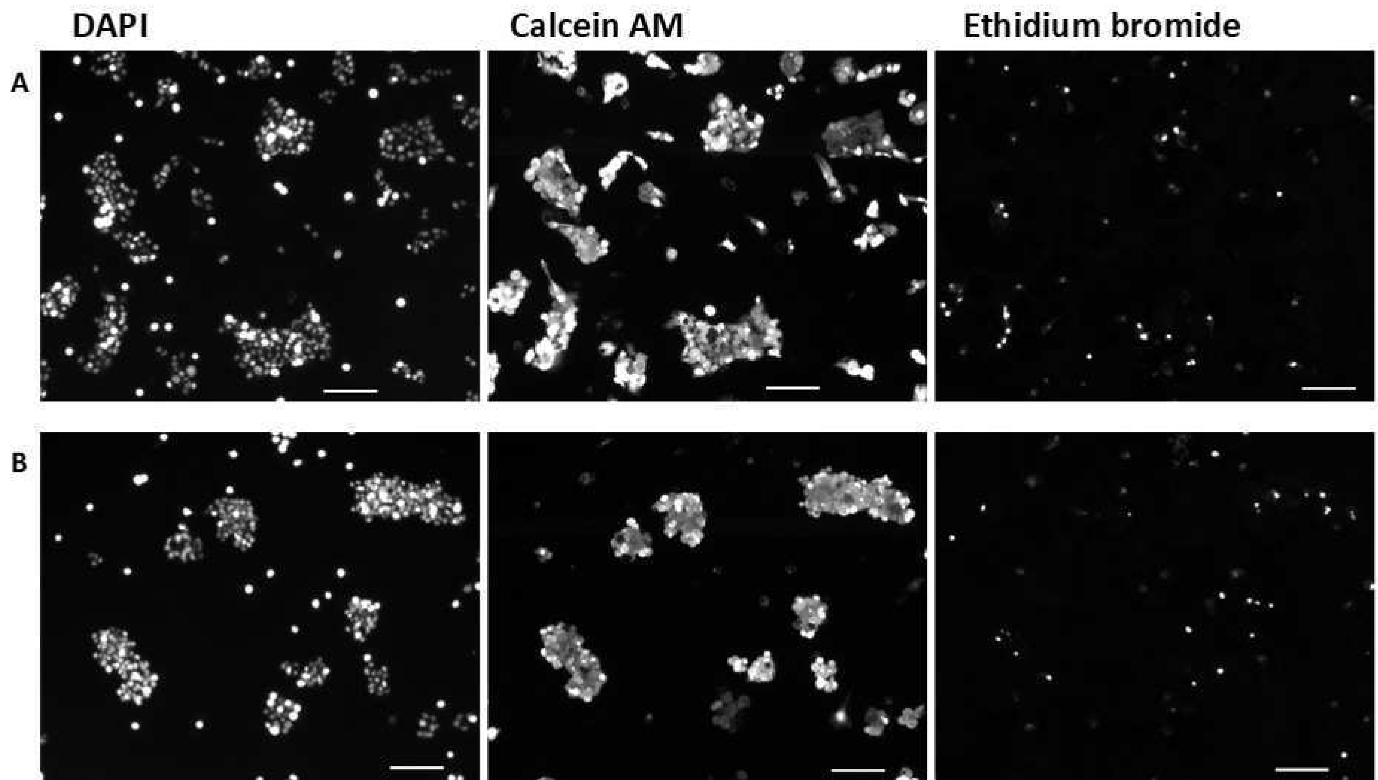
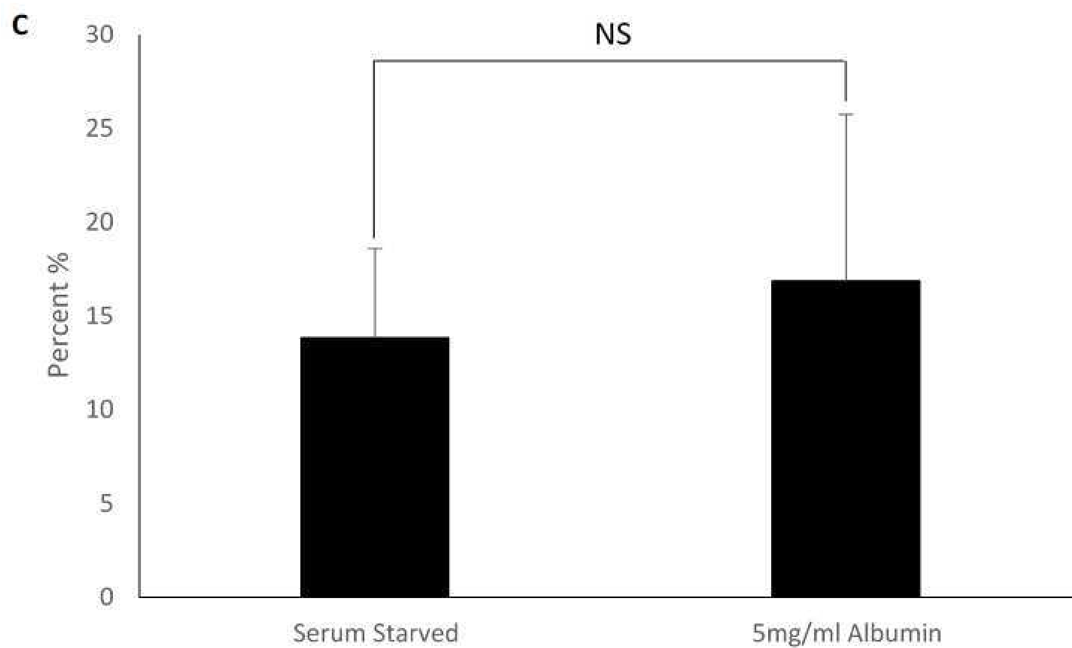

DAPI
Calcein AM
Ethidium bromide
A
B


C
NS
Percent %
30
25
20
15
10
5
0
Serum Starved
5mg/ml Albumin