Albumin promotes proliferation of G1 arrested serum starved hepatocellular carcinoma cells

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Abstract

37
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 into target cells. The
41 immediate effects of albumin on cells however require further understanding. The cell
42 interacting properties and pharmaceutical applications of albumin incentivises continual research
43 into the immediate effects of albumin on cells. The HepG2/C3A hepatocellular carcinoma cell
44 line is 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 observed that the reduced cell counts in serum starved HepG2/C3A
48 cultures were increased by the inclusion of albumin. Cell cycle analysis demonstrated that the
49 percentage of cells in G1 phase during serum starvation was reduced from 86.4 ± 2.3 % to
50 78.3 ± 3.2 % by the inclusion of albumin whereas the percentage of cells in S phase was
51 increased from 6.5 ± 1.5 % to 14.3 ± 3.6 %. A significant reduction in the cell cycle inhibitor
52 protein, P21, accompanied the changes in the proportions of cell cycle phases upon treatment
53 with albumin. We have also observed that the levels of dead cells determined by DNA
54 fragmentation and membrane permeabilization caused by serum starvation (TUNEL: 16.6 ± 7.2
55 %, ethidium bromide: 13.8 ± 4.8 %) were not significantly altered by the inclusion of albumin
56 (11.6 ± 10.2 %, ethidium bromide: 16.9 ± 8.9 %). Therefore, the increase in cell number was
57 mainly caused by albumin promoting proliferation rather than protection against cell death.
58 These primary findings demonstrate that albumin has immediate effects on HepG2/C3A
59 hepatocellular carcinoma cells. These effects should be taken into consideration when studying
60 the effects of albumin bound drugs or pathological ligands bound to albumin on HepG2/C3A
61 cells.
62

Introduction

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

77   cells whereas modified albumin (by surface adsorption to colloidal Au or maleic anhydride
78   treatment) binds gp18 and gp30 and is internalised by endocytosis to be delivered to lysosomes
79   for degradation (Schnitzer et al., 1992; Schnitzer & Bravo, 1993). Another study demonstrates
80   neonatal Fc receptor (FcRn) in transporting albumin across endothelial cells. Recombinant
81   albumin was engineered to have high or low affinity to bind FcRn. Albumin with high affinity
82   was recycled whereas low affinity albumin underwent lysosomal degradation (Schmidt et al.,
83   2017). These cellular interactions with albumin demonstrate that cells selectively dictate the fate
84   of albumin based on the albumin's ligand conformation. This raises the question of whether the
85   cells in turn respond physiologically to albumin.
86   Several studies have indeed demonstrated that different cell types not only respond to albumin,
87   but they respond differently. One of the prominent effects of albumin on cells can be
88   demonstrated by albumins role in apoptosis. Albumin has shown to protect against apoptosis
89   during serum starvation in several cell types including endothelial cells (Zoellner et al., 1996),
90   Pheochromocytoma cells (Zhang et al., 2012) and Neuroblastoma cells (Gallego-Sandín et al.,
91   2005). Albumin also protects against ROS activated apoptosis in chronic lymphocytic leukaemia
92   cells (Moran et al., 2002) and hybridoma T cells (Liu et al., 2012). Albumin can also have a
93   detrimental role, such as that in proximal tubular cells, by causing endoplasmic reticulum stress
94   that consequently leads to apoptosis (Ohse et al., 2006). For its beneficiary role, albumin is used
95   as a supplement in serum free media (Jäger, Lehmann & Friedl, 1988; Francis, 2010) implying
96   its importance in cell maintenance. These effects of albumin however need further interpretation.
97   It has become evident that the diversity of cellular responses to albumin is not only dependent on
98   the cell type but also on the properties of the interacting albumin. This diversity was
99   demonstrated in rat kidney, human squamous carcinoma and various human neuronal cells
100   having responses to fraction V albumin (HPLC fraction of albumin that contains impurities,
101   mainly fatty acids) that are different from their responses to fatty acid free albumin (Keenan et
102   al., 1997; Hooper, Taylor & Pocock, 2005). Effects of albumin on hepatocellular carcinoma cell
103   line have been previously studied demonstrating conflicting outcomes. While one study
104   suggested that albumin stimulates proliferation of hepatocellular carcinoma cells that were
105   inhibited by fatty acids (Lystad et al., 1994), other studies suggested hepatocellular carcinoma
106   cells cease proliferation in response to albumin (Nojiri & Joh, 2014; Bağırsakçı et al., 2017).
107   From the collectively reviewed experiments, it can be extrapolated that the diverse cellular
108   responses to albumin are attributed to the varying albumin receptors in the cell as well as the
109   varying ligand profiles of albumin.
110   Although, studies demonstrated diverse responses of cellular interactions with albumin, more
111   research is still needed to understand the specifics of these cellular responses. Here we study the
112   effect of albumin on HepG2/C3A hepatocellular carcinoma cell line, a contact inhibited subclone
113   of the HepG2 cell line (Aden et al., 1979) that retains some physiological functions of normal
114   hepatocytes (Kelly et al., 1992; Nibourg et al., 2012). The HepG2/C3A cells are used as a model
115   cell line for studying parenchymal biosynthesis (Knowles, Howe & Aden, 1980; Zannis et al.,
116   1981; Nibourg et al., 2012) and screening for cytotoxicity of drug compounds for side effects

117 involving liver injury (Gaskell et al., 2016; Doß et al., 2017). The cell line has also been
118 considered for testing clinical samples in screening for diseases (Sauer et al., 2012, 2018).
119 Furthermore, the HepG2/C3A cell line has been used in clinical trials as a therapeutic in an
120 extracorporeal bioartificial liver device (Nibourg et al., 2012).
121 Since hepatocytes play an important role in albumin metabolism and albumin bound drug
122 clearance (Meijer & van der Sluijs, 1989), we decided to evaluate the HepG2/C3A cell line's
123 immediate responses to albumin. This simple approach is required to understand basic responses
124 of HepG2/C3A cells to albumin prior to engaging in complex experiments in order to exploit
125 these features for testing albumin bound drugs or effects of different bound albumin profiles on
126 cells. Therefore, we applied charcoal defatted human serum albumin to cultured HepG2/C3A
127 cells in the absence of serum. The immediate responses of the cells, particularly proliferation and
128 cell death, were examined. Although similar studies have been previously performed on the
129 HepG2 hepatocellular carcinoma parent clone demonstrating that albumin prevents proliferation
130 (Nojiri & Joh, 2014; Bağırsakçı et al., 2017), experiments in this study were carried out on the
131 HepG2/C3A subclone and were contradictory to these earlier findings. Since HepG2/C3A cell
132 line is commonly used as a model for drug testing (Gaskell et al., 2016; Doß et al., 2017), it is
133 justifiable to have a basic understanding of the effects of albumin on HepG2/C3A cells when
134 testing albumin bound drugs. This primary approach offers a platform and a control method
135 when studying the effects of drug bound albumin and pathologically modified albumin on cell
136 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^{TM}). 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. S1 and Fig. S2) (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 wavelength 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 wavelength of 488nm using the FACSVerse flow
183 cytometer (BD Biosciences).
184 **Immunoblotting**
185 Cells collected after 72 hour treatments were lysed in RIPA buffer (Millipore) containing
186 protease inhibitor cocktail (cOmplete, Roche). Protein concentrations were measured
187 colorimetrically using Pierce BCA protein assay kit (Thermo Scientific). Total protein
188 concentrations of 15 µg for cyclin D1 and 40 µg for p21 in loading buffer (Roti-Load 1, Carl
189 Roth) were separated by SDS-PAGE in a 12% separating gel and 5% stacking gel (Rotiphorese
190 NF-Acrylamide/Bis-solution 30 % (29:1), Carl Roth). Proteins were transferred to PVDF
191 membrane (Immobilon P, Millipore) followed by blocking (Roti-Block, Carl Roth), incubation in
192 primary antibody and then secondary antibody. Rabbit monoclonal to p21 [EPR3993] (1:1000,
193 ab109199, Abcam), Rabbit monoclonal to cyclin D1 [EPR2241] (1:3000, ab134175, Abcam) and
194 Rabbit polyclonal to GAPDH (1:1000, ab9485, Abcam) were used as primary antibodies. The
195 secondary antibody used was HRP conjugated Goat F(ab')2 Anti-Rabbit IgG (1:7500, ab6013,

196 Abcam). The membrane was washed between incubations with TBST (20 mM Tris-HCl (pH
197 7.6), 0.137 M NaCl, 0.05% Tween 20). The blots were then developed using Pierce ECL plus
198 (Thermo Scientific) and imaged using Fusion FX (Vilber). The blots were analyzed
199 densitometrically using ImageJ (https://imagej.nih.gov).

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

209 **Statistics**
210 Four biological replicates were used in each treatment unless stated otherwise. F test was used to
211 determine equal variances. Two tailed T-test assuming equal or unequal (Welch test) variances
212 was carried out accordingly to determine statistical significance when comparing two groups.
213 One way-ANOVA followed by Bonferroni's post hoc analysis was carried out when comparing
214 multiple groups against the control. P value of less than 0.05 is considered significant (α=0.05).
215

216 # Results
217 **Albumin alters cell morphology and results in increased cell counts of serum starved**
218 **HEPG2/C3A cells**
219 To test the immediate effects of albumin, cells were cultured in serum free media containing
220 5mg/ml albumin and compared with the cells cultured in serum free media without albumin
221 (serum starved control). Cells cultured in serum free media containing 5 mg/ml dextran, with a
222 comparable molecular weight to albumin (70 kDa), was carried out as a control for effects that
223 might relate to oncotic pressure.
224 Serum starved controls displayed a slight irregular morphology but retained the epithelial
225 polygonal shape and remained as monolayers in colonies. Noticeable morphological differences
226 were observed in albumin treated cells when compared to the serum starved control. Cells
227 remained in colonies but displayed a rounded morphology, formed clusters and grew in more
228 than one layer. Dextran treated cells demonstrated a morphological effect more in resemblance to
229 serum starved cells and did not exhibit the rounded morphology, clusters and layering seen in
230 albumin treated cells (Fig. 1).
231 The effects of albumin on total cell counts of serum starved cells was evaluated in comparison to
232 Dextran treated cells and serum starved control. Data passed equal variance test and were
233 analysed by one-way ANOVA followed by Bonferroni's multiple comparison versus control
234 (serum starved cells). Serum starvation for 72 hours yielded an average total cell count of
235 2.8×10^6 cells. Inclusion of albumin in serum starved cells cultured for 72 hours significantly

236 increased the average total cell count to 4x10^6 cells (p=0.019, n=4, α=0.05). Dextran treatment of
237 serum starved cells had fewer cells (2x10^6 cells) than the serum starved control but the difference
238 was not significant (p=0.14, n=4, α=0.05) (Fig. 1). These findings suggest that albumin alters cell
239 morphology and results in increased total cell counts in serum starved cells. These effects are not
240 simply due to osmotic pressure and are specific for albumin as these results could not be
241 reproduced in cultures treated with an equal molecular weight of Dextran. Serum starved
242 HepG2/C3a cells demonstrated a similar response of increased total cell counts when higher
243 doses of albumin (25 mg/ml and 50 mg/ml) were included in the cultures (Fig. S3).

244 **Albumin promotes proliferation but does not prevent cell death in serum starved**
245 **HEPG2/C3A cells**

246 Albumin triggered increased cell counts in serum starved HEPG2/C3A cells requires further
247 explanation into whether these effects are a result of promoting proliferation or preventing cell
248 loss. HEPG2/C3A cells cultured in media containing 10% FBS were maintained for 120 hours
249 until confluent (Fig. S4) and possibly contact inhibited (Kelly et al., 1992; Davis, Ho & Dowdy,
250 2001; Cho et al., 2005). Cell cycle analysis demonstrated that the cells cultured in 10% FBS for
251 120 hours were 86.6 % in G1 phase, 6.2 % in S phase and 7.2 % in G2/M phase (Fig. S4). The
252 confluent cells were harvested then seeded at the regular seeding density (2x10^6 cells) in serum
253 starved media and serum starved media containing 5mg/ml albumin. Cell cycle analysis after 72
254 hours in culture demonstrated a significant reduction in G1 phase in albumin treated cells
255 (78.3±3.2 %) compared to serum starved cells (86.4±2.3 %) (equal variance t.test, p=0.006, n=4,
256 α=0.05). Whereas S phase was significantly higher in albumin treated cells (14.3±3.6 %)
257 compared to serum starved cells (6.5±1.5 %) (unequal variance (Welch) t.test, p=0.016, n=4,
258 α=0.05). There was however no significant difference in G2/M phase between albumin treated
259 cells (7.4±1.9 %) and serum starved cells (7±2.2 %) (equal variance t.test, p=0.8, n=4, α=0.05)
260 (Fig. 2 and Fig. S1). These results suggest that serum starvation resulted in a cell cycle arrest in
261 G1 phase, while inclusion of albumin promoted cell cycle transition into S phase.
262 Western blot analysis demonstrates that levels of p21 (average relative density) expressed in
263 serum starved cells (1±0.64) is significantly reduced (equal variance t.test, p=0.034, n=4,
264 α=0.05) by the inclusion of 5 mg/ml albumin (0.12±0.08). Levels of cyclin D1 between serum
265 starved cells (1±0.72) and albumin treated cells (1.07±0.87) were not significantly different
266 (equal variance t.test, p=0.0, n=3, α=0.05) (Fig. 2). This suggests that albumin promotes cell
267 cycle progression in serum starved cells by suppressing p21.
268 Serum starvation causes cell apoptosis and necrosis in cultured cells. The effects of serum
269 starvation are however slow in HepG2 cells (Zhuge & Cederbaum, 2006; Liang et al., 2013).
270 This slow response is recapitulated in HEPG2/C3A cells (Fig. S2 and Fig. S5); hence, the 72-
271 hour time point was selected for this study. Apoptosis and necrosis were measured in
272 HEPG2/C3A cells by TUNEL assay and ethidium bromide staining respectively. Serum starved
273 cells were 16.6 ± 7.2 % apoptotic and albumin treated cells were 11.6 ± 10.2 % apoptotic (Fig.
274 3). Although TUNEL positive cells were perceptibly lower in some of the samples when treated

275 with albumin (Fig. S6), the effect was not statistically significant (unequal variance (Welch)
276 t.test, n=4, p=0.46, α=0.05) (Fig. 3).
277 Cells in culture for 72 hours were stained with calcein AM (green) and ethidium bromide (red) to
278 demonstrate presence of live and necrotic cells respectively (Decherchi, Cochard & Gauthier,
279 1997). DAPI (blue) nuclear stain was carried out to quantify total number of cells in the field of
280 view. Live cells were characterised by esterase activity that causes calcein AM to fluoresce in the
281 cytoplasm and is clearly present in all treatments. Necrotic cells were characterised by damaged
282 cell membrane that is permeable to ethidium bromide allowing it to stain the nucleus (Fig. 4).
283 Serum starvation resulted in 13.8 ± 4.8 % necrosis. Inclusion of 5mg/ml albumin resulted in an
284 increase in necrosis to 16.9 ± 8.9 % but is not significantly higher than serum starved cultures
285 (equal variance t.test, n=4, p=0.5, α=0.05) (Fig. 4). Cells grown in 10% FBS were used as
286 negative controls for TUNEL assay (0.8 ± 0.2 % apoptotic) and ethidium bromide staining ($1.7 \pm$
287 0.8% necrotic) (Fig. S7). While inclusion of albumin significantly accounts for an increase in
288 cell counts and proportion of cells in S phase during serum starvation it does not significantly
289 protect against cell death by either apoptosis or necrosis.

Discussion and conclusions

291 The frequent interactions of naturally abundant albumin with cells in the body as well as its wide
292 exogenous applications in the biomedical and pharmaceutical industries (Dennis et al., 2002;
293 Sleep, Cameron & Evans, 2013) prompted us to study the effects of albumin on cells. We have
294 selected HepG2/C3A carcinoma cells because they originate from hepatocytes (Aden et al.,
295 1979) and have been used as a model for studying hepatocellular carcinoma (Chen et al., 2015;
296 Ao et al., 2017) as well as normal hepatocyte functions (Nibourg et al., 2012; Gaskell et al.,
297 2016). Hepatocytes play an important role in the clearance of albumin bound substances (Meijer
298 & van der Sluijs, 1989), therefore some of these albumin interacting properties can potentially be
299 used in targeting hepatocellular carcinoma or studying the effects of pathologically modified
300 albumin on cellular function. This study aims to determine immediate effects of albumin on
301 HepG2/C3A cell proliferation and death during serum starvation. Here the effects of albumin
302 free from ligands is carried out to pave the way for understanding how albumin bound ligands
303 may allosterically affect the cell. There have been several studies (mentioned in the introduction)
304 describing the receptor interactions of a variety of cells with albumin dependent on the albumin's
305 ligand profile and how these cells determine the fate of this albumin molecule dependent on its
306 ligand profile. Most of these studies describe the effects of cells on albumin but studies on the
307 effects of albumin on cells are lacking. Hence, we carried out these experiments.
308
309 Although, the effects of albumin on the proliferation of hepatocellular carcinoma cells have been
310 previously studied, the outcomes of those studies are debatable. They argued that albumin
311 inhibits tumour evidenced by its increased levels correlating with cancer remission and addition
312 of albumin to serum starved hepatoma cells caused G1 arrest (Nojiri & Joh, 2014; Bağırsakçı et
313 al., 2017). It is worth noting that serum is essential for hepatocellular carcinoma growth (Zhuge
314 & Cederbaum, 2006; Liang et al., 2013) whereas serum starvation is routinely carried out to

315 arrest cells in G1 phase (Davis, Ho & Dowdy, 2001; Langan, Rodgers & Chou, 2017). These
316 observations that albumin causes cell cycle arrest are possibly a result of an artefact.
317 We carried out our experiments on HEPG2/C3A cells, a selected colony of HepG2 cells, that are
318 responsive to contact inhibition. In our approach, the cells were collected for experimentation
319 after they were grown to confluency to synchronize them by means of contact inhibition (Kelly
320 et al., 1992; Davis, Ho & Dowdy, 2001; Cho et al., 2005). Cells were then grown under serum
321 starved conditions with or without albumin. The initial observed effects of albumin on serum
322 starved HEPG2/C3A cells were morphological. Serum starved cells retain epithelial morphology
323 and ability to spread. Albumin caused cells to appear more round, small and clustered (Fig. 1).
324 The cells were then counted and analysed for cell cycle after 72-hour treatments. The reason for
325 selecting this time point is that during serum starvation, earlier time points did not demonstrate
326 sufficient cell cycle arrest whereas at later time points, the cells were mostly dead. At 72 hours
327 after serum starvation we observed minimal cell loss (mean number of cells harvested were not
328 lower than cells seeded) and sufficient G1 cell cycle arrest was observed (Fig. S2). With the
329 inclusion of albumin, we observed an increase in cell counts that correlated with a reduction in
330 percentages of cells in G1 phase and an increase in S phase compared to serum starved controls.
331 Furthermore, we investigated levels of cell cycle promotor protein cyclin D1 and cell cycle
332 inhibitor protein p21 (Reed et al., 1994) in serum starved cells and albumin containing cultures.
333 Results demonstrate that inclusion of albumin in serum starved cultures significantly reduces P21
334 but does not significantly alter levels of Cyclin D1. This suggests that albumin permits cell cycle
335 transition from G1 to S phase by downregulating p21 which results in increased cell
336 proliferation.
337
338 To further rule out proliferation inhibitory effects of albumin that were suggested in the studies
339 mentioned above (Nojiri & Joh, 2014; Bağırsakçı et al., 2017), we treated HEPG2/C3A cells
340 grown in media containing FBS with 10mg/ml albumin and did not observe a significant change
341 in cell counts (equal variance t.test, n=3, p=0.36) (Fig. S8).
342
343
344 Cell death was measured to rule out a protective effect of albumin. TUNEL assay was carried out
345 to determine cells that underwent DNA fragmentation, an indicator of apoptosis (Crowley,
346 Marfell & Waterhouse, 2016). Nuclear staining of cells with ethidium bromide was carried out to
347 demonstrate membrane damage as a result of necrosis or secondary necrosis (Zong & Thompson,
348 2006). Inclusion of albumin in serum starved cells did not significantly change the proportion of
349 dead cells identified by these methods. This suggest that albumin does not protect against DNA
350 fragmentation and membrane permeabilization that occurs during serum starvation.
351 We have argued that albumin has an immediate effect on cell proliferation by promoting cell
352 cycle transition from G1 to S phase in the absence of serum and other proteins including growth
353 promoting factors. However, we did not study whether albumin directly interfered with
354 molecular pathways of transcription factors involved in cell cycle transition or indirectly

355 influenced proliferation through interfering with other processes such as cell spreading. A deeper
356 understanding of the mechanism of albumin´s effect on cell proliferation requires more detailed
357 studies. This can include identification of receptors and downstream signalling pathways
358 triggered by albumin-receptor interactions.
359 We investigated modes of cell death individually, but we did not examine any overlaps between
360 DNA fragmentation and membrane permeabilization. Furthermore, we did not examine whether
361 the underlying cause of death in the membrane permeabilized cells was apoptosis or direct
362 necrosis. The tests simply suggest that albumin did not prevent various fates of cell death that
363 occur during serum starvation.
364 These findings argue that albumin resulted in increased cell counts as a result of increased
365 proliferation through promotion of G1 to S phase transition and not by prevention of cell death in
366 serum starved HepG2/C3A cells. This study offers primary results and a platform for further
367 investigations into the molecular interactions of albumin with cells. Additionally, this approach
368 can be used as a control to study different modifications of albumin, ligand profiles and drug
369 bound albumin.
370

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377

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Figure 1

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

Albumin alters morphology of serum starved HepG2/C3A cells demonstrated in 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) Albumin results in increased total cell counts after 72 hours in culture. Values are mean ± SD (n=4). *$p < 0.05$, NS= not significant.
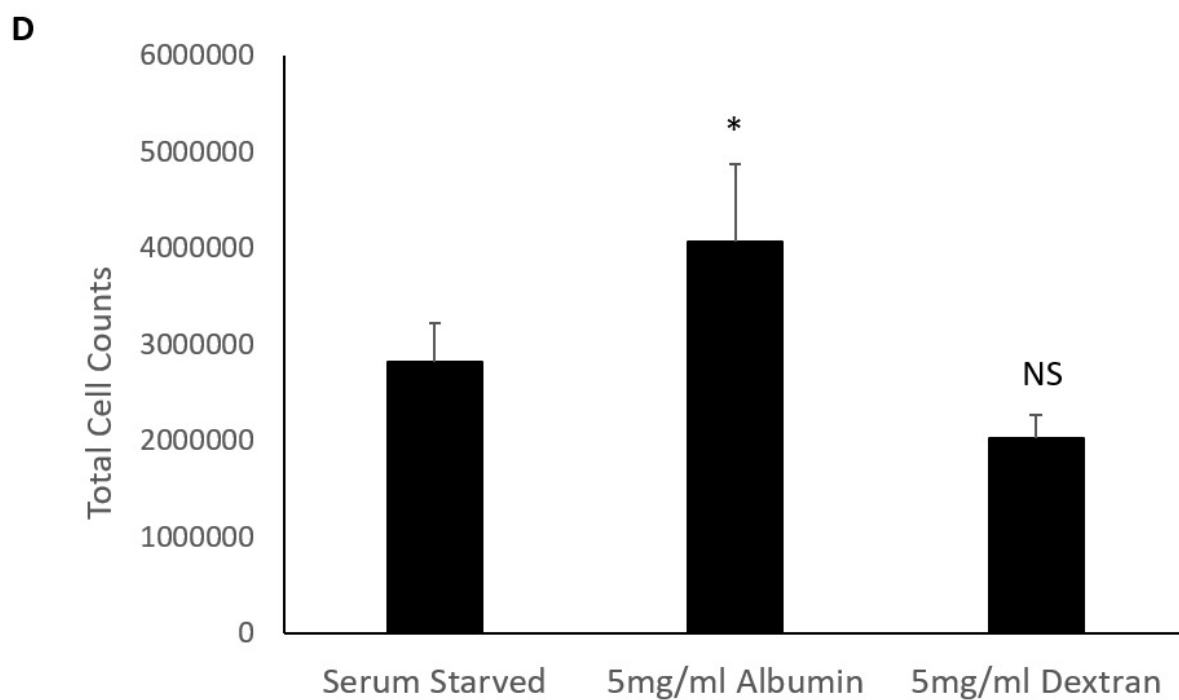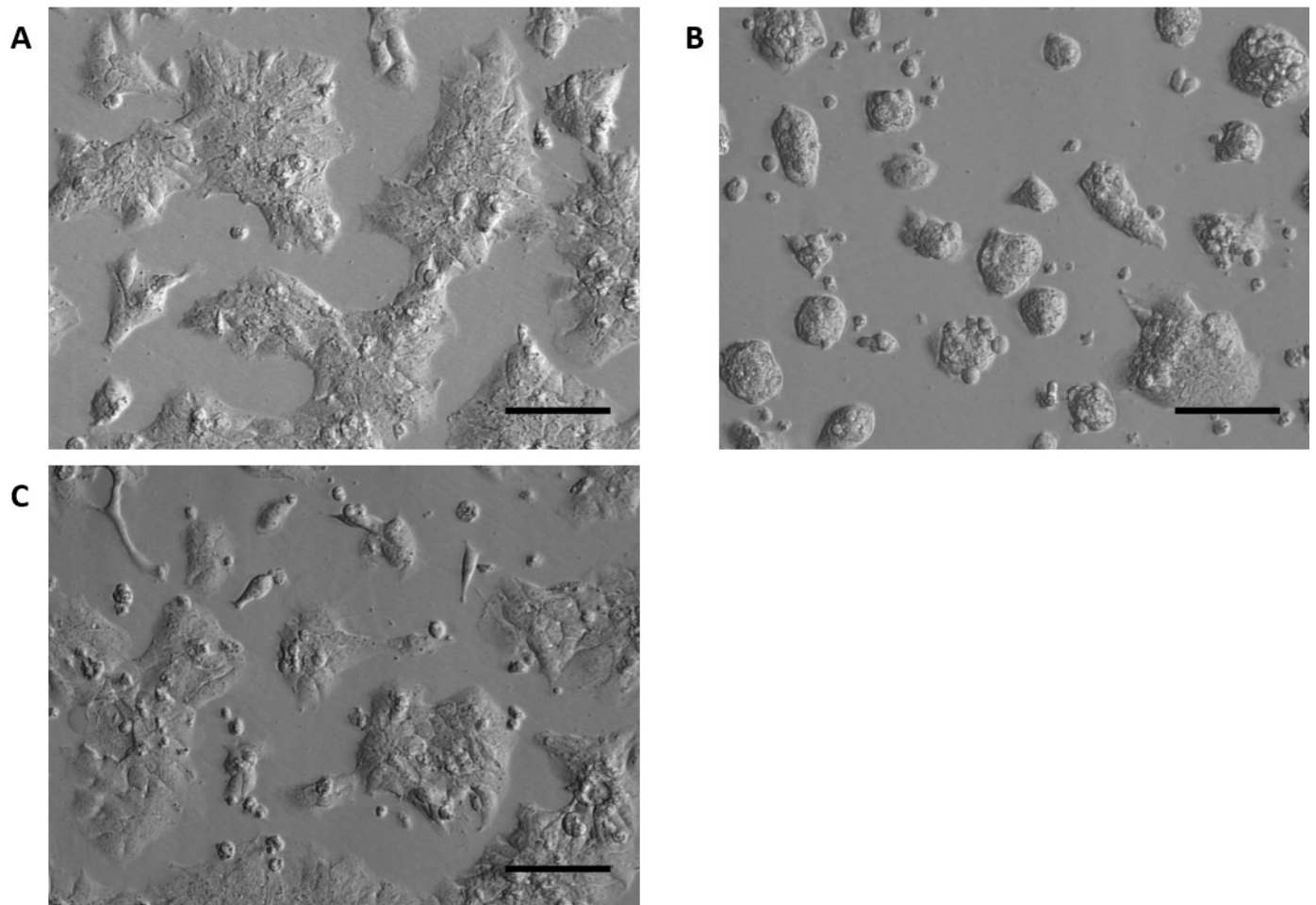
Figure 2

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 5 mg/ml albumin. (C) Percentage of cell cycle stages calculated from the histogram using the Watson pragmatic algorithm shows a decrease in G1 phase and an increase in S phase in albumin containing media. Values are mean ± SD (n=4). *p < 0.05, **p<0.01, NS= not significant. (D) Representative images of western blots demonstrating significantly reduced P21 levels but no significant change in cyclin D1 levels in albumin containing cultures compared to serum free controls.
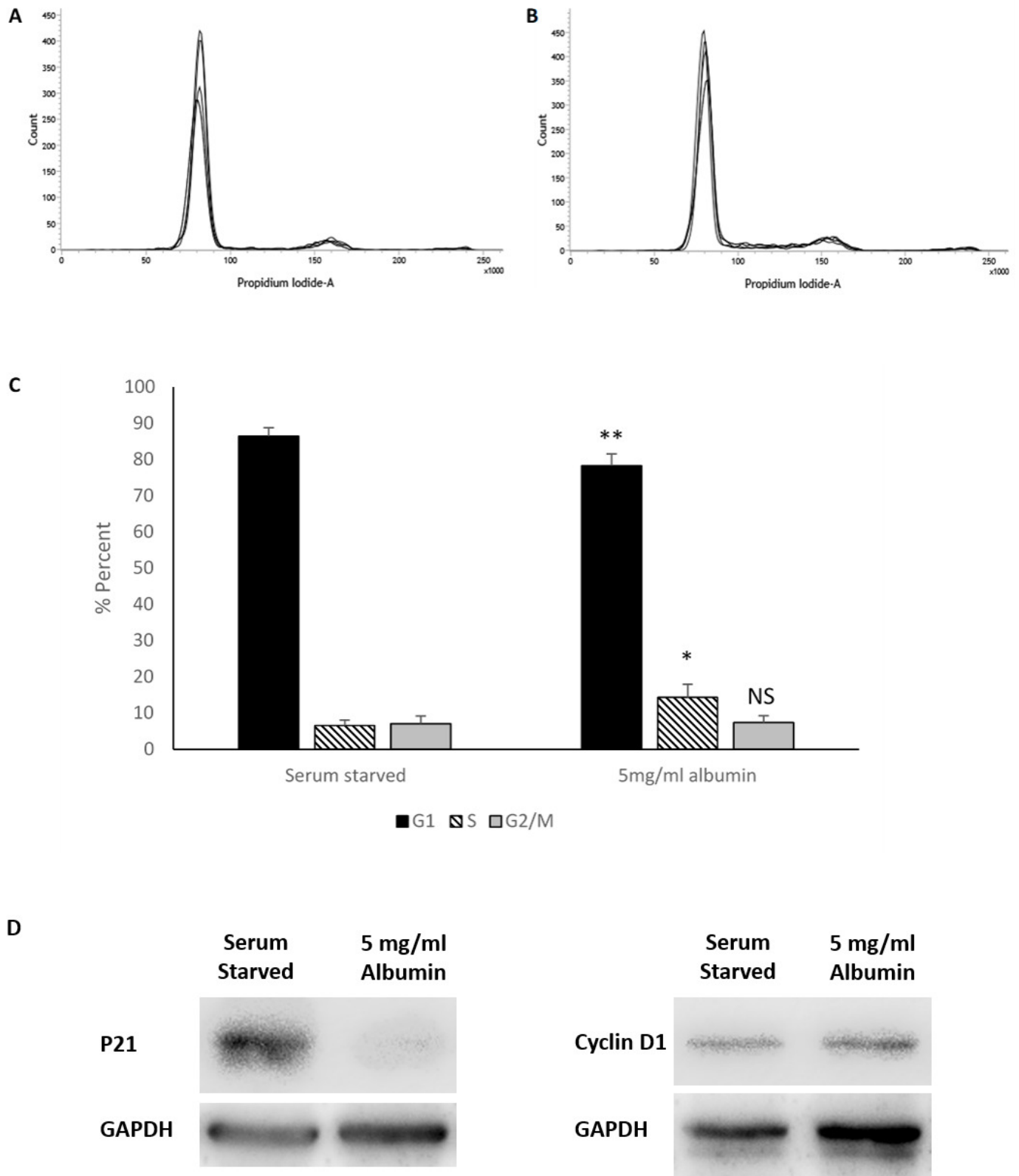
Figure 3

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

Percentages of apoptotic cells were not significantly different demonstrated in flowcytometry overlay of dot plots of TUNEL positive cells (red) and negative cells (black) of 72 hour cultures of HepG2/C3A 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.

A

FITC-A

10^5
10^4
10^3
10^2
0

0   50   100 150 200 250
                              x1000
SSC-A

Apoptosis

B

FITC-A

10^5
10^4
10^3
10^2
0

0   50   100 150 200 250
                              x1000
SSC-A

Apoptosis

C

Percent %

25
20
15
10
5
0

Serum starved   5mg/ml Albumin

NS
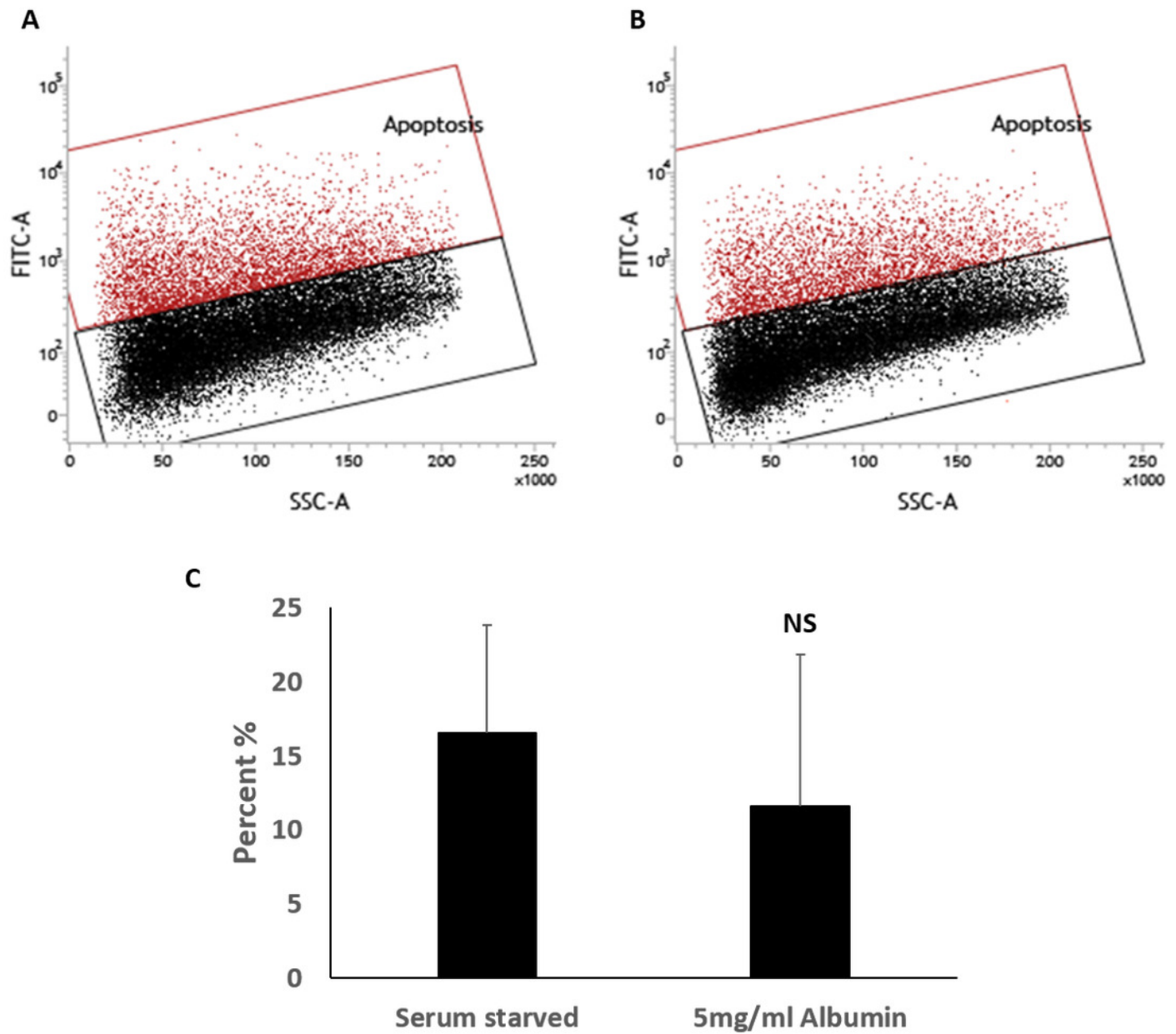
Figure 4

Live dead fluorescent micrographs of cells.

Percentages of dead cells were not significantly different demonstrated by fluorescence microscopy images 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) Chart demonstrating differences in the percentage of ethidium bromide stained cells between treatments. Values are mean ± SD (n=4). NS= not significant.

DAPI   **Calcein AM**   **Ethidium bromide**

A

B

C

% Ethidium Bromide Staining

NS

30

25

0

Serum Starved   5mg/ml Albumin