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Collagen overlays can inhibit leptin and adiponectin secretion but not lipid accumulation in adipocytes

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Background. White adipose tissue (WAT) is essential for energy storage as well as being an active endocrine organ. The secretion of adipokines by adipocytes can affect whole body metabolism, appetite, and contribute to overall health. WAT is comprised of lipid-laden mature adipocytes, as well as immune cells, endothelial cells, pre-adipocytes, and adipose-derived stem cells. In addition, the presence of extracellular matrix (ECM) proteins in WAT can actively influence adipocyte differentiation, growth, and function. Type I collagen is an abundant fibrous ECM protein in WAT that is secreted by developing adipocytes. However, the extent and overall effect of Type I collagen on adipokine secretion in mature adipocytes when added exogenously has not been established. **Methods.** We characterized the effects of Type I collagen overlays prepared using two different buffers on adipocyte physiology and function when added at different times during differentiation. In addition, we compared the effect of collagen overlays when adipocytes were cultured on two different tissue culture plastics that have different adherent capabilities. Triglyceride accumulation was analyzed to measure adipocyte physiology, and leptin and adiponectin secretion was determined to analyze effects on adipokine secretion. **Results.** We found that collagen overlays, particularly when added during the early differentiation stage, impaired adipokine secretion from mature adipocytes. Collagen prepared using PBS had a greater suppression of leptin than adiponectin while collagen prepared using HANKS buffer suppressed the secretion of both adipokines. The use of CellBind plates further suppressed leptin secretion. Triglyceride accumulation was minimally impacted with any of the collagen overlays. **Discussion.** Adipokine secretion can be selectively altered by collagen overlays. Thus, it is feasible to selectively manipulate the secretion of adipokines by adipocytes *in vitro* by altering the composition or timing of collagen overlays. The use of this technique could be applied to studies of adipokine function and secretion *in vitro* as well as having potential therapeutic implications to specifically alter adipocyte functionality *in vivo*.

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Collagen overlays can inhibit leptin and adiponectin secretion but not lipid accumulation in adipocytes

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18 **Abstract**

19 **Background.** White adipose tissue (WAT) is essential for energy storage as well as being an
20 active endocrine organ. The secretion of adipokines by adipocytes can affect whole body
21 metabolism, appetite, and contribute to overall health. WAT is comprised of lipid-laden mature
22 adipocytes, as well as immune cells, endothelial cells, pre-adipocytes, and adipose-derived stem
23 cells. In addition, the presence of extracellular matrix (ECM) proteins in WAT can actively
24 influence adipocyte differentiation, growth, and function. Type I collagen is an abundant fibrous
25 ECM protein in WAT that is secreted by developing adipocytes. However, the extent and overall
26 effect of Type I collagen on adipokine secretion in mature adipocytes when added exogenously
27 has not been established.

28 **Methods.** We characterized the effects of Type I collagen overlays prepared using two different
29 buffers on adipocyte physiology and function when added at different times during
30 differentiation. In addition, we compared the effect of collagen overlays when adipocytes were
31 cultured on two different tissue culture plastics that have different adherent capabilities.
32 Triglyceride accumulation was analyzed to measure adipocyte physiology, and leptin and
33 adiponectin secretion was determined to analyze effects on adipokine secretion.

34 **Results.** We found that collagen overlays, particularly when added during the early
35 differentiation stage, impaired adipokine secretion from mature adipocytes. Collagen prepared
36 using PBS had a greater suppression of leptin than adiponectin while collagen prepared using
37 HANKS buffer suppressed the secretion of both adipokines. The use of CellBind plates further
38 suppressed leptin secretion. Triglyceride accumulation was minimally impacted with any of the
39 collagen overlays.

40 **Discussion.** Adipokine secretion can be selectively altered by collagen overlays. Thus, it is
41 feasible to selectively manipulate the secretion of adipokines by adipocytes *in vitro* by altering
42 the composition or timing of collagen overlays. The use of this technique could be applied to
43 studies of adipokine function and secretion *in vitro* as well as having potential therapeutic
44 implications to specifically alter adipocyte functionality *in vivo*.

45 **Introduction**

46 White adipocytes serve a crucial function in storing free fatty acids in the form of
47 triglycerides (TG) for energy use (Berry et al., 2013) and to prevent toxic deposition of free fatty
48 acids in ectopic sites (Bays, Mandarino & Defronzo, 2004). Mature, lipid-laden adipocytes
49 develop from fate-committed pre-adipocytes through the activation of a series of well-regulated
50 transcription factors culminating in the increased expression of CCAAT/enhancer-binding
51 protein alpha (C/EBP-α) and peroxisome proliferator-activator receptor gamma (PPAR-γ), often
52 termed as the "master regulators" of adipogenesis. Increased expression of C/EBP-α and PPAR-γ
53 promote the expression of key proteins essential for lipid and carbohydrate storage proteins, such
54 as glucose transporter 4 (Glut4) and perilipin (Plin1), allowing the formation of large lipid
55 droplets that store TG and cholesteryl esters, which are a primary phenotype of terminally
56 differentiated mature adipocytes (Berry et al., 2013).

57 In addition to its energy storing role, white adipose tissue (WAT) can modulate numerous
58 tissues via secretion of adipokines. Key adipokines include adiponectin, which is inversely
59 associated with obesity and increased with fasting, and leptin, which is elevated in obese
60 individuals and decreased with fasting (Stern, Rutkowski & Scherer, 2016). Both adiponectin
61 and leptin are secreted from terminally differentiated adipocytes, and they act on WAT as well as
62 distal tissues. For example, adiponectin enhances insulin sensitivity and induces the expansion of
63 WAT, which prevents the toxic deposition of free fatty acids in other organs (Yamauchi et al.,
64 2001, 2002; Berg et al., 2001; Xu et al., 2003) ,whereas leptin acts through the sympathetic
65 nervous system to induce lypolysis of WAT (Zeng et al., 2015). In contrast, both adiponectin and
66 leptin can promote glucose uptake by skeletal muscle (Tomas et al., 2002; Bates et al., 2002).
67 Moreover, leptin promotes the proliferation of breast cancer cells (Ray, Nkhata & Cleary, 2007;

68 Soma et al., 2008; Dubois et al., 2014) while adiponectin inhibits their proliferation (Li et al.,
69 2011). Thus, adipokines secreted by WAT have multiple and varied effects on selected tissues
70 and cells.

71 The role of the extracellular environment on adipocyte function remains incompletely
72 understood (Huang & Greenspan, 2012; Poulos et al., 2015). Adipocytes are supported by
73 extracellular matrix (ECM) proteins, where laminin, fibronectin, and collagen types I-VI are the
74 major constituents with the precise ECM composition differing between species and WAT
75 depots (Mariman & Wang, 2010). Adipocytes express and secrete ECM proteins, and collagen
76 synthesis during early adipogenesis may promote adipocyte differentiation. Different densities of
77 ECM proteins can cause adipocytes that are grown on ECM scaffolds to alter the *in vitro*
78 secretion of adipokines such as adiponectin and MCP-1 (Li et al., 2010). In addition, pre-
79 adipocytes can actively remodel the ECM via the secretion of matrix metalloproteinases
80 (Christiaens et al., 2008).

81 Systems to model the ECM-adipocyte interactions using collagen-embedded pre-
82 adipocytes or adipocytes for biological study or as a means to engineer tissue for engraftment
83 have been described; however, they are often complicated, require specialized equipment, or do
84 not model the *in vivo* ECM (Von Heimburg et al., 2003; Stacey et al., 2009; Chun & Inoue,
85 2014). Here, one of our aims was to establish a technique that is simpler than embedding
86 adipocytes within a three-dimensional matrix, would maintain the pre-adipocyte/adipocyte
87 interaction with the ECM, and would still allow for co-culture analysis with other cell types in
88 the future studies. We used this technique to examine the effect of Type I collagen, a highly
89 abundant protein in adipocyte ECM (Mariman & Wang, 2010) that is commercially available,
90 and was previously used with success for adipocyte cultures (Von Heimburg et al., 2003; Stacey

91 et al., 2009; Chun & Inoue, 2014). We found a differential effect of the collagen preparation on
92 TG accumulation, and leptin or adiponectin secretion that was further affected by timing and the
93 type of tissue culture (TC) plastic used. These findings suggest that minor manipulations to the
94 ECM surrounding adipocytes can selectively affect their physiology or function.
95

96 **Materials and Methods**

97 *Cells and Adipogenesis assay*

98 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA)
99 and confirmed to be free of mycoplasma contamination using the MycoAlert assay (Lonza,
100 Basel, Switzerland). All media and supplements were obtained from Invitrogen Life
101 Technologies (Waltham, MA, USA) unless otherwise indicated. Cells were maintained in high-
102 glucose (25 mM) DMEM supplemented with 10% newborn calf serum, 1%
103 penicillin/streptomycin, and 1% sodium pyruvate (DMEM/NCS). Cells were cultured in TC
104 treated 24-well plates (Falcon cat. no. 353226) or CellBind treated 24-well plates (Corning cat.
105 no. 3337). As shown in Fig. 1 and as previously described (Smith et al., 2015), pre-adipocytes
106 were plated at 5 x 10^4 cells per well to ensure 100% confluency after 24and allowed to undergo
107 cell-contact dependent growth arrest for further 24 h. At this time, media was replaced with
108 growth media [high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1%
109 penicillin/streptomycin and 1% sodium pyruvate (DMEM/FBS)] containing 0.5 mM 3-isobutyl-
110 1-methylxanthine (IBMX) and 1 μM Dexamethasone (Dex) (Millipore, Billerica, MA, USA) and
111 cultured for 48 h. Then the media was replaced with DMEM/FBS containing 10 μg/ml insulin
112 (Sigma-Aldrich, St. Louis, MO, USA) and cultured for an additional 48 h. Media were then

113 changed to DMEM/FBS and cells cultured for up to 5 additional days with media replacement
114 every 48 h with DMEM/FBS.
115
116 *Collagen matrices*
117 FibriCol® collagen (cat. no. 5133-A) was obtained from Advanced BioMatrix Inc (Carlsbad,
118 CA, USA). The collagen was diluted to 3.3 mg/mL in PBS (1.9 mM NaH_2PO_4, 8.4 mM
119 Na_2HPO_4, 137 mM NaCl, pH 7.2) or in 1X HANKS buffer (1.26 mM Ca_2Cl_2, 0.81 mM $MgSO_4$,
120 5.4 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.34 mM Na_2HPO_4, 5.5 mM D-glucose, 0.05
121 mM Phenol Red Sodium Salt, obtained as 10X concentrate cat. no. 0919101-54, MP
122 Biomedicals, Santa Ana, CA, USA). Collagen in PBS (PBS-collagen) was neutralized to pH 7-
123 7.5 by addition of HCl prior to layering onto cells. Collagen in HANKS was neutralized to pH 7-
124 7.5 by addition of 250 mM HEPES (pH 7.2) (HANKS-collagen). Collagen solutions or control
125 solutions lacking collagen (300 µL) were gently layered on top of the adipocytes and allowed to
126 polymerize at 37˚C for 40 min, thus generating a collagen layer of an approximate thickness of
127 1.5 mm. For adipocytes not treated with collagen or buffer controls at that particular stage,
128 medium was not removed. At the end of the 40 min, after the collagen solutions were
129 polymerized, 1 mL of medium, appropriate for the stage of differentiation, was layered on top of
130 the collagen layer or buffer solution (Fig. 1). At day 2, the medium overlaid was DMEM/FBS
131 with IBMX/Dex (DMEM/FBS+IBMX/Dex). At day 4, the medium overlaid was DMEM/FBS
132 with insulin (DMEM/FBS+Insulin). At day 6, the medium overlaid was DMEM/FBS and
133 replaced with DMEM/FBS every 48 h.
134
135 *Triglyceride quantification*

136 Media were removed at day 12 (Fig. 1); cells and collagen was washed twice with PBS and then
137 removed by scraping followed by re-suspension in 1 mL PBS. Lipids from the re-suspended cells
138 were extracted using the Bligh-Dyer method (Bligh & Dyer, 1959), followed by additional
139 manipulations as follows. Following the removal of the organic solvent layer from the Bligh-
140 Dyer extraction, lipids remaining in the upper phase were re-extracted (in duplicate) by adding
141 2.5 mL of chloroform, vortexing for 30 s, and centrifuging at 1000 *g* for 5 min. The organic
142 solvent layer was removed and pooled with the organic solvent layer from the Bligh-Dyer
143 extraction. The pooled extracts were back extracted by mixing with an equal volume of PBS,
144 vortexing for 30 s, and centrifuging at 1000 *g* for 5 min. The organic solvent layer was removed,
145 dried under N_2(g), re-suspended in 500 µL isopropanol, and stored under N_2(g) at -20˚C until
146 needed. To control for extraction efficiency, 10 µg of a TG standard (cat. no. 17810, Sigma
147 Aldrich, St. Louis, MO, USA) was extracted as above, and the TG quantified with 25 µL of
148 extracted sample (n=11) was compared to the TG quantified from 25 µL of 0.02 µg/µL TG
149 standard (in isopropanol). TG cellular accumulation was corrected for extraction efficiencies
150 within each extraction procedure (mean extraction efficiency was 88.2% with a range of 79.8% -
151 97.7%). A colorimetric commercial kit from Wako Diagnostics (Richmond, VA, USA) was used
152 to quantify TG, using a standard curve of 0-50 µg TG.
153
154 *Adiponectin and leptin quantification*
155 Mouse leptin and adiponectin concentrations in the culture supernatant were determined using
156 DuoSet ELISA kits specific for mouse from R&D Systems (Minneapolis, MN, USA) following
157 the manufacturer's instructions. Samples were analyzed in duplicate. A 4-parameter log-logistic
158 model was used to fit the data to the standard curves run simultaneously using R v3.0 (R Core

159 Team, 2015). Calibration curves were run to ensure accurate dilution of the supernatants and
160 found to require dilution of 1024-fold for adiponectin and no dilution for leptin. Samples where
161 all replicates from one treatment group had an absorbance below background are indicated as not
162 detected (ND). For samples with levels of leptin or adiponectin below the level of detection, the
163 amount was set to 8 pg/mL for leptin and 0.035 ng/mL for adiponection, which was just below
164 the lowest detectable concentration of 8.9 pg/mL and 0.039 ng/mL, respectively. Analysis of
165 differences were performed using Wilcoxon rank-sum analysis as samples with undetectable
166 levels can be included without compromising the analysis.
167
168 *Statistical analysis*
169 Statistical analysis was performed in R v3.0 (R Core Team, 2015), as indicated in the respective
170 figure legends. Experiments were repeated at least three times beginning with a unique passage
171 of 3T3-L1 cells. Each independent experiment is considered a biological replicate. Differences
172 were considered significant at $P<0.05$.
173
174 **Results**
175
176 **Collagen matrices influence adipocyte function**
177 We found that there was a statistically significant decrease in overall TG accumulation
178 with collagen overlays when assessed at day 12 (Fig. 2A). However, when we analyzed the
179 effects of the overlay at each timepoint, we found that there was no significant effect of PBS-
180 collagen on the total TG accumulation at any specific stage. In contrast, addition of HANKS-

181 collagen on day 2, but not day 4 or 6, resulted in a statistically significant reduction in total TG
182 (Fig. 2A).

183 We then analyzed leptin secretion at days 9 (Fig. 2B) and 12 (Fig. 2C), and we compared
184 the amount of leptin detected in the supernatant when collagen overlays were added at day 2, 4,
185 or 6. Overall, we found that the addition of either PBS-collagen or HANKS-collagen had an
186 overall effect of suppressing leptin accumulation when analyzed at both days 9 and 12. The
187 suppression was greater when the collagen was added at day 2 or day 4 compared to when added
188 at day 6 with no leptin detected after collagen addition in most cases (Fig. 2B-C). Addition of
189 collagen at day 6 did not significantly affect the amount of leptin detected at day 9 but
190 suppressed the amount of leptin detected at day 12 with HANKS-collagen only. At day 9, the
191 overall effect of PBS-collagen was statistically different from HANKS-collagen with statistically
192 similar effects at day 12.

193 Adiponectin secretion was affected more by HANKS-collagen at both day 9 (Fig. 2D)
194 and 12 (Fig. 2E) when compared to PBS-collagen. Addition of HANKS-collagen, but not PBS-
195 collagen at day 2 resulted in a large and significant inhibition of adiponectin secretion at day 9
196 (Fig. 2D). By day 12, adiponectin levels remained significantly lower in adipocytes with
197 HANKS-collagen added at day 2 or 4, but not at day 6, suggesting the effect is time- or
198 maturation-dependent (Fig. 2E). Addition of PBS-collagen significantly, but modestly,
199 suppressed adiponectin secretion by day 12 and at day 9 when added at day 4 only.

200

201 **Effect of CellBind plates on adipocyte physiology and function in the presence of collagen**

202 The previous experiments were performed using typical TC treated plastic. However,
203 culturing 3T3-L1 cells on specially treated dishes with higher levels of incorporated oxygen on

204 the plastic, branded CellBind (Pardo et al., 2010), can increase the ease of the adipogenesis assay
205 because the cells remain more firmly attached. This effect is noticed particularly after IBMX and
206 Dex treatment at day 2 when the cells have a more rounded morphology (S.L. Christian and N.K.
207 Pallegar, unpublished observations). Therefore, we sought to determine if the addition of
208 collagen overlays in combination with CellBind plates would affect adipogenesis.

209 We first determined if CellBind plates affected adipocyte physiology or function in the
210 absence of collagen. As expected, we found a significant increase in the amount of cellular TG,
211 as well as a significant increase of secreted leptin and adiponectin, when cells were induced to
212 undergo adipogenesis compared to control (Neg) cells (Fig. 3A-C). We observed a trend towards
213 an increase in TG accumulation when adipogenesis was induced in the CellBind plates compared
214 to regular TC plates (P=0.07). Overall, there was no significant effect of plate type on leptin or
215 adiponectin secretion. However, we observed spontaneous release of adiponectin on CellBind
216 plates from control cells (Neg) resulting in no significant increase in adiponectin secretion
217 detected at day 9 in response to adipogenesis induction (Pos) (Fig. 3C).

218 We next determined the effect of the collagen overlays on TG accumulation and
219 adipokine secretion when CellBind plates were used. Similar to the regular TC plates, we found
220 an overall suppression of TG accumulation in the presence of the collagen overlays (Fig. 4A).
221 However, the only significantly different pairwise comparison was with PBS-collagen added at
222 day 4. There was no overall significant difference between PBS-collagen and HANKS-collagen
223 on TG accumulation.

224 In contrast, we found that leptin levels on day 9 were particularly disrupted when cells
225 were treated with either buffer alone or collagen in either type of buffer during adipogenesis
226 (Fig. 4B), suggesting that leptin secretion from cells grown on CellBind plates is more sensitive

227 to any environmental disruption during adipogenesis, which occurs in both conditions. However,
228 by day 12 the amount of leptin detected from the cells without collagen was similar to cells
229 grown without disruption (compare Fig. 4C to Fig. 3B). Addition of HANKS-collagen at day 4,
230 but not day 2 or 6, resulted in a significant decrease in leptin accumulation at day 12 (Fig. 4C). In
231 addition, there was a significant overall difference between the addition of PBS-collagen
232 compared to HANKS-collagen in leptin accumulation at day 12, with HANKS-collagen having a
233 greater impact.

234 There was an overall significant decrease in adiponectin accumulation at days 9 and 12
235 with the addition of collagen (Fig. 4D-E). Overlaying HANKS-collagen at day 4 resulted in a
236 significant decrease in adiponectin detected at both day 9 and day 12. Overlaying PBS-collagen
237 at day 4 caused a significant decrease when detected at day 12 but not day 9. Moreover, the
238 magnitude of the decrease in adiponectin accumulation was much less than with TC plates
239 (compare Fig 4D-E to Fig. 2D-E).

240

241 **Discussion**

242 Our goal was to determine if collagen matrix overlays would impact adipocyte
243 physiology, as measured by TG accumulation, and function, as measured by adipokine secretion.
244 We found that both physiology and function could be differentially affected by the addition of
245 collagen overlays in a manner that depended on when during adipogenesis the collagen was
246 added. Moreover, collagen overlays prepared using different buffers had differential effects on
247 adiponectin and leptin secretion.

248 Overall TG accumulation was generally suppressed by the addition of both PBS-collagen
249 and HANKS-collagen (Fig. 2A and 4A). However, only two timepoints showed a specific

250 reduction in TG accumulation when compared to without buffer alone, with neither of the
251 changes particularly substantial. Therefore, we conclude that the collagen matrix overlays do not
252 substantially alter the normal accumulation of lipid in mature adipocytes. It remains to be
253 determined if the modest decreases in TG accumulation would translate to biologically relevant
254 outcomes in an *in vivo* situation.

255 Our analysis of adipocyte function as determined by adipokine secretion revealed that
256 leptin and adiponectin can be affected by both the method of collagen matrix preparation and the
257 type of TC plastic used. We do not believe that the reduction in adipokine detection is due to
258 collagen physically blocking the passage of secreted proteins that are subsequently detected by
259 ELISA. Leptin has a calculated mass of approximately 19 kDa protein while adiponectin has a
260 calculated mass of 26 kDa. Since leptin was impacted more than adiponectin, it is unlikely that
261 the collagen matrices are reducing the passage of secreted proteins based on size. Adiponectin is
262 glycosylated in at least 6 sites (Richards et al., 2006, 2010), whereas post-translational
263 modifications of leptin, with the exception of disulfide bonds, have not been reported. The
264 predicted pI of leptin is 5.85, while the predicted pI of unmodified adiponectin is 5.57,
265 suggesting that different charges on the unmodified proteins of -2.5 and -6.4, respectively, could
266 potentiate charge-charge interactions between the adipokine and collagen. In fact, an interaction
267 between adiponectin and collagen has been reported previously *in vitro* and in injured but not
268 healthy blood vessels *in vivo* (Okamoto et al., 2000). However, leptin has not been reported to
269 interact with collagen. Moreover, equivalent amounts of leptin and adiponectin were secreted
270 when the collagen was added to more mature cells (i.e. at day 6). Thus, there is no evidence to
271 suggest that the collagen matrices physically impede the release of adipokines into the
272 surrounding media.

273 The adipocytes were exposed to an absence or reduction of glucose during the 40 min
274 collagen polymerization period for PBS and HANKS-based buffers, respectively. Reduced
275 adipogenesis is observed when cells are exposed to low glucose levels during early phases of
276 differentiation, corresponding to days 2-5 in our study, but not at later stages (Jackson et al.,
277 2017). The levels of TG, adiponectin, and leptin secreted by cells exposed to no/low glucose
278 buffer were similar to the controls by day 12 but generally lower at day 9 (compare Fig. 2 to
279 Figs. 3 and 4). Therefore, it appears that the cells are able to overcome any effects of the short-
280 term low glucose exposure given enough time for full differentiation and maturation. However,
281 differentiation of cells in the CellBind plate when combined with the 40 min buffer or collagen
282 incubation further reduced leptin secretion, particularly at day 9, suggesting that firm binding of
283 the cells to the plates affects their ability to withstand other stressors, such as reduced glucose
284 levels. Therefore, it is possible that the combination of reduced glucose in the presence of
285 monomeric or polymerized collagen is necessary for the full reduction in adipocyte function.
286 It is possible that, even after addition of media with sufficient glucose levels, the collagen
287 layers could impact nutrient or oxygen availability to the cells due to physical interference by
288 advective mixing. Unfortunately, it was not possible to control for this as the buffer solutions,
289 used as controls for the absence of media during polymerization, do not form discrete layers. In
290 fact, the buffer dilutes the media by ~30% thus potentially decreasing the nutrient availability as
291 well as the adipokine concentration compared to the wells with polymerized collagen. Therefore,
292 we may be underestimating the reduction in adipokine or TG secretion. Future studies will be
293 required to determine the concentration of key nutrients and oxygen in the collagen layer or at
294 the collagen-adipocyte interface in comparison to the buffer controls to determine if this could be
295 causing the effects we observed.

296 The collagen overlays could also decrease the clonal expansion that occurs during
297 adipogenesis of 3T3-L1 cells (Gregoire, Smas & Sul, 1998; Tang, Otto & Lane, 2003). This re-
298 entry into the cell cycle after contact-dependent growth arrest occurs during the early phases of
299 adipogenesis and could therefore be impacted by addition of collagen at day 2 or 4. If this were
300 the case, we could expect a significant reduction in TG accumulation as, in theory, there would
301 be a reduction of up to 50% of the cells. Since we have not observed a significant or substantial
302 reduction in TG accumulation for most of the pairwise analysis, we do not believe that this is the
303 major mechanism. However, precise analysis of DNA replication will be required to
304 conclusively establish if the collagen overlays affect clonal expansion.

305 Leptin was strongly affected by the addition of both types of collagen matrices.
306 Strikingly, addition of collagen at the earlier stage of adipogenesis (day 2 or day 4) resulted in
307 leptin secretion that was, in many cases, below the level of detection. Thus, addition of collagen
308 during active differentiation decreases leptin secretion in a manner that is not readily restored by
309 additional time in culture. Leptin expression is regulated by a variety of transcription factors
310 including C/EBPα, SREBP1, and FosL1 during adipogenesis (Miller et al., 1996; Mason et al.,
311 1998; Wrann & Rosen, 2012). In addition, leptin expression is promoted by insulin,
312 glucocorticoids, and even leptin itself (Wrann & Rosen, 2012). The ability of collagen or buffer
313 to impair leptin secretion when added at early stages of adipogenesis but not late stages suggests
314 that these manipulations may be interfering with the action of a regulator that acts early during
315 adipogenesis. Elucidating the mechanism for this stage-dependent effect will be an important
316 focus for future study.

317 In contrast to leptin, effects of collagen overlays on adiponectin secretion were modest.
318 PBS-collagen had very little impact on adiponectin secretion while HANKS-collagen, especially

319 when associated with TC-treated plates measured at day 9, substantially decreased the secretion
320 of adiponectin. Adiponectin expression is regulated by insulin via activation of PPAR-γ as well
321 as by other transcription factors such as C/EBPα and negatively regulated by FoxO1 (Shehzad et
322 al., 2012). Thus, it appears that addition of HANKS-collagen significantly impairs the regulation
323 of adiponectin synthesis and/or secretion, but this effect is reduced when cells are cultured in
324 CellBind plates. While it is clear that collagen overlays differentially regulate the appearance of
325 adiponectin and leptin, the precise mechanism for this regulation will require further study.

326 It is not clear why the HANKS-collagen had different effects than PBS-collagen on
327 adipokine secretion, when compared to their respective buffer controls. While we observed that
328 the HANKS-collagen solidified at a slower rate than PBS-collagen, both were solidified
329 completely within 24 h. The HANKS buffer contains potassium ions, $CaCl_2$, $MgSO_4$, and D-
330 glucose, which are absent in the PBS-collagen preparation. The differences in buffer
331 compositions did not cause obvious changes to the collagen overlays at the macroscale (data not
332 shown). We have not found any published studies on the specific interactions between these
333 molecules and collagen that may explain the differing cellular responses. Therefore, future study
334 will be necessary to dissect out the key factors that cause the functional changes to the collagen
335 matrices.

336 Regardless of the mechanism, we have clearly shown that manipulations to the
337 preparation of Type I collagen can result in significant and specific alterations to adipocyte
338 physiology and function. Thus, selective alteration of adipokine secretion without impacting TG
339 storage may be possible using collagen matrices. Adapting this method to *in vivo* procedures
340 could potentially be used to modify appetite-regulating hormones without diminishing the

341 essential role of adipocytes to store free fatty acids, which are toxic in high abundance or when
342 deposited ectopically (Bays, Mandarino & Defronzo, 2004).
343
344 **Conclusions**
345 In summary, we found that the secretion of leptin and adiponectin can be selectively
346 manipulated while not substantially impairing TG synthesis in developing adipocytes by use of
347 different collagen preparations and cell culture plates. These findings may provide researchers a
348 new way to affect adjacent cells or tissue by selectively manipulating adipocyte function *in vitro*
349 or, potentially *in vivo*.
350
351
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354
355

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357

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460

461 **Figure Legends**

462

463 **Fig. 1: Schematic diagram of 3T3-L1 adipogenesis and timing of collagen overlays.** Pre-
464 adipocytes were cultured for 24 h on regular tissue-culture (TC) treated or CellBind plates, until
465 they underwent contact-dependent growth arrest. Collagen, or buffer only control, was overlaid
466 at days 2, 4, or 6 and allowed to solidify for 40 min at 37˚C. Medium was overlaid onto the
467 collagen or buffer after the 40 min polymerization period. DMEM/FBS medium containing
468 IBMX and dexamethasone (DMEM/FBS+IBMX/Dex) was added at day 2, DMEM/FBS with
469 insulin (DMEM/FBS+Insulin) was added at day 4, and DMEM/FBS was added at day 6 and
470 replaced every 48 h. Pre-adipocytes, prior to induction of differentiation, are indicated by
471 starburst shapes. Lipid droplets in mature adipocytes are shown with small red circles. Addition
472 of PBS-collagen or HANKS-collagen is indicated at day 2, 4, or 6. Adiponectin and leptin
473 concentrations were determined at day 9 and day 12, and TG accumulation was determined at
474 day 12.

475

476 **Fig. 2: Triglyceride, leptin, and adiponectin production are differentially affected by PBS-**
477 **collagen and HANKS-collagen when cells are plated on regular TC-treated plates.** A) Total
478 TG in 3T3-L1 cells at day 12 after adding collagen overlays at days 2, 4 or 6. B-E) Levels of
479 secreted leptin and adiponectin were determined at day 9 (B,D) and day 12 (C,E). Note that
480 leptin and adiponectin concentrations are shown in Log10 scale as the changes in concentration
481 can span multiple orders of magnitude. Statistical analysis was done by 3-way ANOVA. *A*
482 *priori* analysis of control vs. collagen at each stage by T-test for TG and adiponectin and by
483 Wilcoxon rank sum for leptin levels as some samples were below the detection limit of the

484 ELISA (ND: not detected). *P<0.05, **P<0.01, ***P<0.001, shown are mean ± sem of 3
485 independent biological replicates.
486
487 **Fig. 3: Adipogenesis is not significantly different when 3T3-L1 cells are cultured in regular**
488 **tissue-culture (TC) treated dishes or CellBind dishes.** A) TG accumulation at day 12, B)
489 leptin and C) adiponectin secretion at days 9 and 12 in cells culture on regular TC or CellBind
490 plates in the absence (Neg) or presence (Pos) of adipogenic inducers as described in the methods,
491 with no buffer incubation steps. Note that leptin and adiponectin concentrations are shown in
492 Log10 scale as the changes in concentration can span multiple orders of magnitude. Statistical
493 analysis by 2-way ANOVA. *A priori* analysis of control vs. collagen at each stage by T-test for
494 TG and adiponectin and by Wilcoxon rank sum for leptin levels as some samples were below the
495 detection limit of the ELISA. *P<0.05, **P<0.01, ***P<0.001. Mean ± sem shown of 3-4
496 independent biological replicates.
497
498 **Fig. 4: Triglyceride (TG), leptin, and adiponectin production are differentially affected by**
499 **PBS-collagen and HANKS-collagen when cells are plated on CellBind plates.** A) Total TG in
500 3T3-L1 cells at day 12 after adding collagen overlays at days 2, 4 or 6. B-E) Levels of secreted
501 leptin and adiponectin were determined at day 9 (B, D) and day 12 (C, E). Note that leptin and
502 adiponectin concentrations are shown in Log10 scale as the changes in concentration can span
503 multiple orders of magnitude. Statistical analysis by 3-way ANOVA.. *A priori* analysis of control
504 vs. collagen at each stage by T-test for TG and adiponectin and by Wilcoxon rank sum for leptin
505 levels as some samples were below the detection limit of the ELISA (ND: not detected).
506 *P<0.05, **P<0.01, ***P<0.001. Mean ± sem of 3 independent biological replicates is shown.

Figure 1

Schematic diagram of 3T3-L1 adipogenesis and timing of collagen overlays

Pre-adipocytes were cultured for 24 h on regular tissue-culture (TC) treated or CellBind plates, until they underwent contact-dependent growth arrest. Collagen, or buffer only control, was overlaid at days 2, 4, or 6 and allowed to solidify for 40 min at 37˚C. Medium was overlaid onto the collagen or buffer after the 40 min polymerization period. DMEM/FBS medium containing IBMX and dexamethasone (DMEM/FBS+IBMX/Dex) was added at day 2, DMEM/FBS with insulin (DMEM/FBS+Insulin) was added at day 4, and DMEM/FBS was added at day 6 and replaced every 48 h. Pre-adipocytes, prior to induction of differentiation, are indicated by starburst shapes. Lipid droplets in mature adipocytes are shown with small red circles. Addition of PBS-collagen or HANKS-collagen is indicated at day 2, 4, or 6. Adiponectin and leptin concentrations were determined at day 9 and day 12, and TG accumulation was determined at day 12.

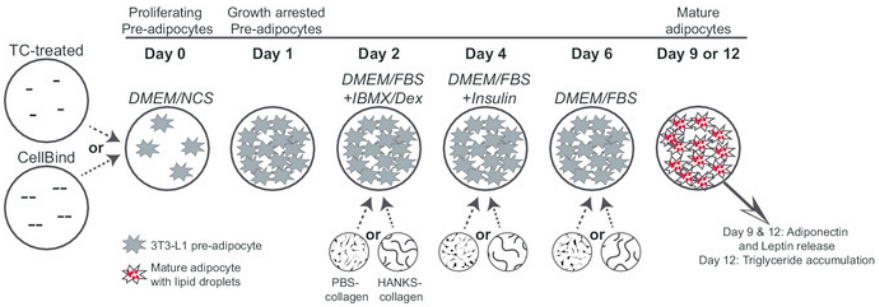
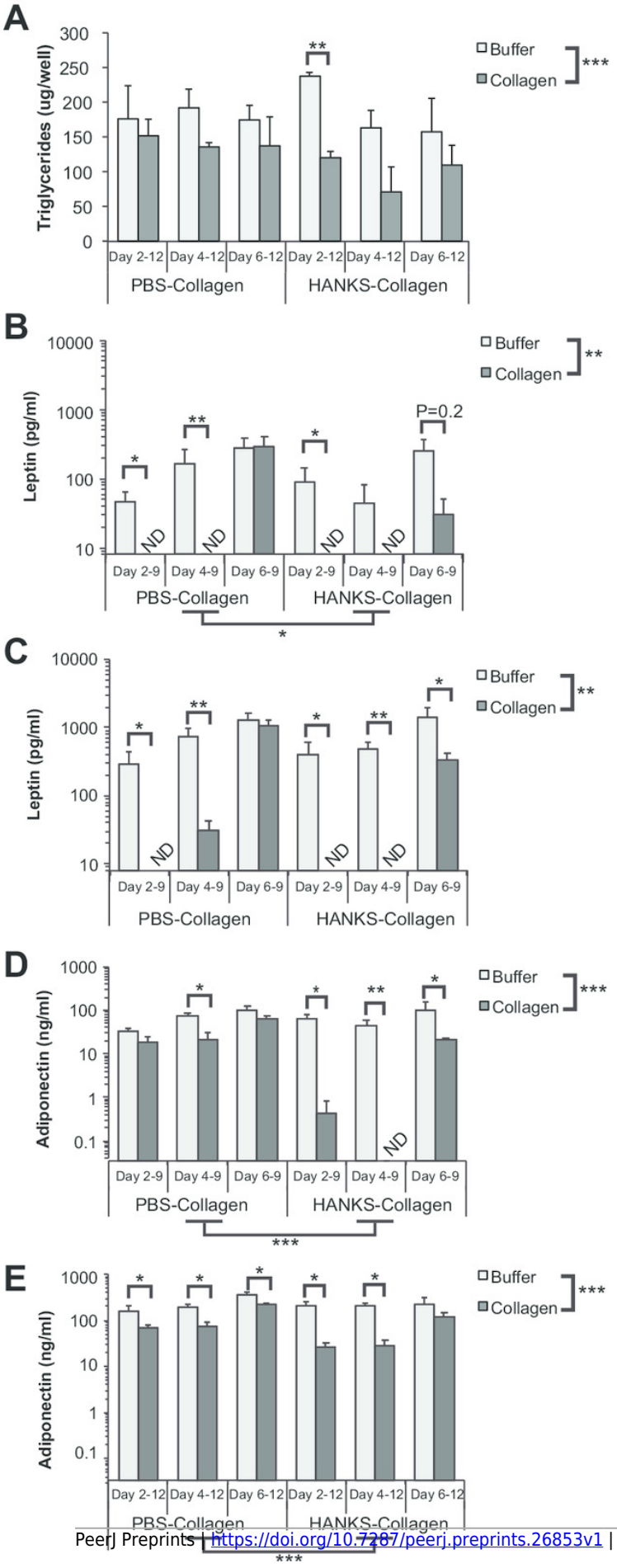


Figure 2

Triglyceride, leptin, and adiponectin production are differentially affected by PBS-collagen and HANKS-collagen when cells are plated on regular TC-treated plates

A) Total TG in 3T3-L1 cells at day 12 after adding collagen overlays at days 2, 4 or 6. B-E) Levels of secreted leptin and adiponectin were determined at day 9 (B,D) and day 12 (C,E). Note that leptin and adiponectin concentrations are shown in Log10 scale as the changes in concentration can span multiple orders of magnitude. Statistical analysis was done by 3-way ANOVA. *A priori* analysis of control vs. collagen at each stage by T-test for TG and adiponectin and by Wilcoxon rank sum for leptin levels as some samples were below the detection limit of the ELISA (ND: not detected). *P<0.05, **P<0.01, ***P<0.001, shown are mean ± sem of 3 independent biological replicates.


A
Triglycerides (ug/well)
300
250
200
150
100
50
0
Buffer
Collagen
***
**
Day 2-12
Day 4-12
Day 6-12
Day 2-12
Day 4-12
Day 6-12
PBS-Collagen
HANKS-Collagen
B
Leptin (pg/ml)
10000
1000
100
10
Buffer
Collagen
**
P=0.2
*
**
*
ND
ND
ND
ND
Day 2-9
Day 4-9
Day 6-9
Day 2-9
Day 4-9
Day 6-9
PBS-Collagen
HANKS-Collagen
*
C
Leptin (pg/ml)
10000
1000
100
10
Buffer
Collagen
**
*
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*
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ND
ND
ND
Day 2-9
Day 4-9
Day 6-9
Day 2-9
Day 4-9
Day 6-9
PBS-Collagen
HANKS-Collagen
D
Adiponectin (ng/ml)
1000
100
10
1
0.1
Buffer
Collagen
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ND
Day 2-9
Day 4-9
Day 6-9
Day 2-9
Day 4-9
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PBS-Collagen
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Adiponectin (ng/ml)
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Day 6-12
PBS-Collagen
HANKS-Collagen
***

Figure 3

Adipogenesis is not significantly different when 3T3-L1 cells are cultured in regular tissue-culture (TC) treated dishes or CellBind dishes.

A) TG accumulation at day 12, B) leptin and C) adiponectin secretion at days 9 and 12 in cells culture on regular TC or CellBind plates in the absence (Neg) or presence (Pos) of adipogenic inducers as described in the methods, with no buffer incubation steps. Note that leptin and adiponectin concentrations are shown in Log10 scale as the changes in concentration can span multiple orders of magnitude. Statistical analysis by 2-way ANOVA. *A priori* analysis of control vs. collagen at each stage by T-test for TG and adiponectin and by Wilcoxon rank sum for leptin levels as some samples were below the detection limit of the ELISA. *P<0.05, **P<0.01, ***P<0.001. Mean ± sem shown of 3-4 independent biological replicates.

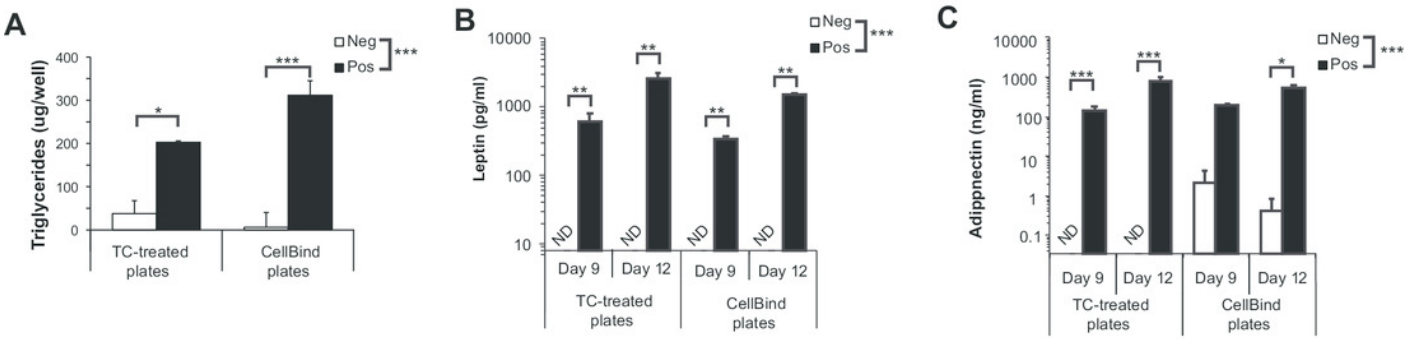
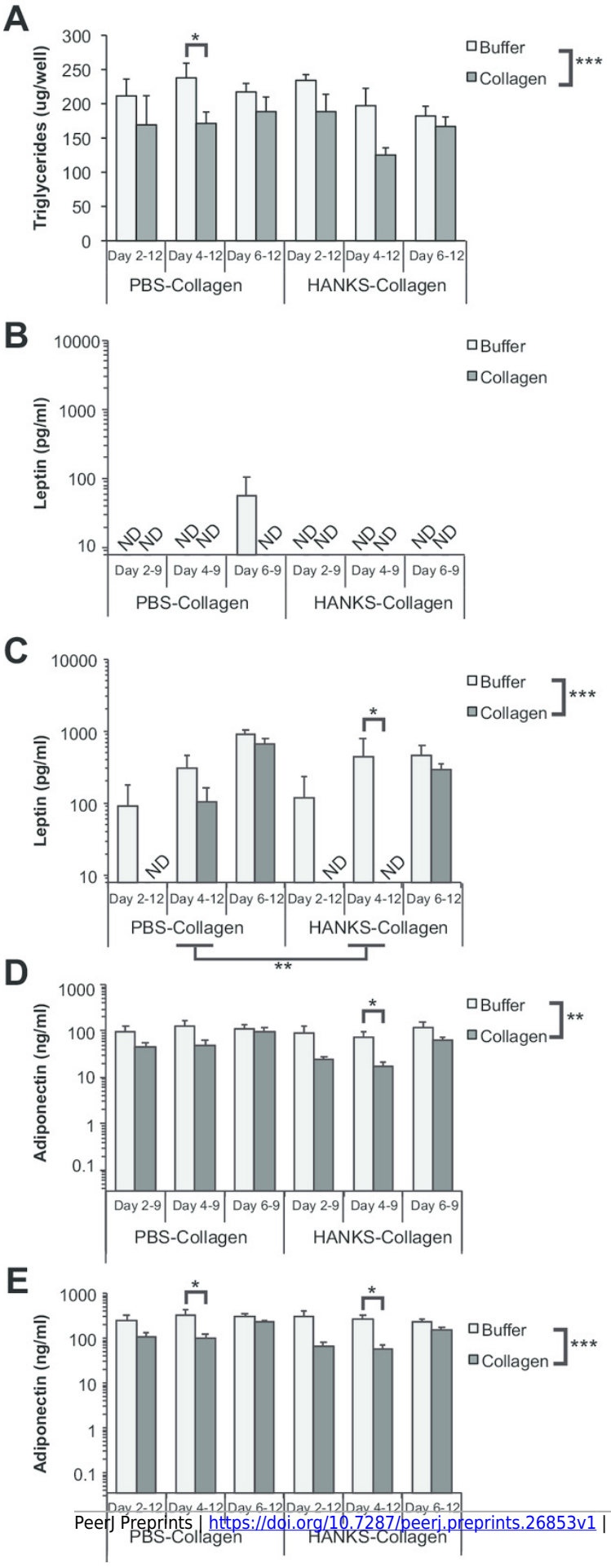


Figure 4

Triglyceride (TG), leptin, and adiponectin production are differentially affected by PBS-collagen and HANKS-collagen when cells are plated on CellBind plate

A) Total TG in 3T3-L1 cells at day 12 after adding collagen overlays at days 2, 4 or 6. B-E) Levels of secreted leptin and adiponectin were determined at day 9 (B, D) and day 12 (C, E). Note that leptin and adiponectin concentrations are shown in Log10 scale as the changes in concentration can span multiple orders of magnitude. Statistical analysis by 3-way ANOVA.. *A priori* analysis of control vs. collagen at each stage by T-test for TG and adiponectin and by Wilcoxon rank sum for leptin levels as some samples were below the detection limit of the ELISA (ND: not detected). *P<0.05, **P<0.01, ***P<0.001. Mean ± sem of 3 independent biological replicates is shown.


A
Triglycerides (ug/well)
300
250
200
150
100
50
0
*
Buffer
Collagen
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Day 2-12
Day 4-12
Day 6-12
Day 2-12
Day 4-12
Day 6-12
PBS-Collagen
HANKS-Collagen
B
Leptin (pg/ml)
10000
1000
100
10
ND
Buffer
Collagen
Day 2-9
Day 4-9
Day 6-9
Day 2-9
Day 4-9
Day 6-9
PBS-Collagen
HANKS-Collagen
C
Leptin (pg/ml)
10000
1000
100
10
ND
*
Buffer
Collagen
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Day 2-12
Day 4-12
Day 6-12
Day 2-12
Day 4-12
Day 6-12
PBS-Collagen
HANKS-Collagen
**
D
Adiponectin (ng/ml)
1000
100
10
1
0.1
*
Buffer
Collagen
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Day 2-9
Day 4-9
Day 6-9
Day 2-9
Day 4-9
Day 6-9
PBS-Collagen
HANKS-Collagen
E
Adiponectin (ng/ml)
1000
100
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0.1
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Buffer
Collagen
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Day 2-12
Day 4-12
Day 6-12
Day 2-12
Day 4-12
Day 6-12
PBS-Collagen
HANKS-Collagen