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1 ***Short communication***

2 **Title: Use of MALDI-TOF mass spectrometry to explore the peptidome and proteome of**
3 **in-vitro produced bovine embryos pre-exposed to oviduct fluid.**

4 **Authors:** Charles Banliat^{a,b}, Valérie Labas^{a,c}, Daniel Tomas^{a,c}, Ana-Paula Teixeira-Gomes^{c,d},
5 Benoît Guyonnet^b, Pascal Mermillod^a and Marie Saint-Dizier^{a,e*}

6 ^aCNRS, IFCE, INRAE, Université de Tours, PRC, 37380, Nouzilly, France;

7 ^bUnion Evolution, Noyal-Sur-Vilaine, France;

8 ^cINRAE, Université de Tours, CHU de Tours, PIXANIM, 37380 Nouzilly, France;

9 ^dINRAE, Université de Tours, ISP, 37380 Nouzilly, France

10 ^eTours University, Tours, France

11 *Corresponding author (marie.saint-dizier@univ-tours.fr)

12

13 **Competing interest:** none.

14 **Short title:** MALDI-TOF MS analysis of cattle embryos.

15 **Abstract.** In order to identify oviduct fluid (OF) peptides and proteins possibly uptaken by
16 developing embryos, *in-vitro* produced bovine embryos exposed or not to OF were
17 individually analyzed by MALDI-TOF mass spectrometry. Overall, 11 masses were
18 overabundant in OF-treated embryos compared to controls, among which one at 8.9 kDa
19 annotated as immediate early response 3-interacting protein 1 or a peptide of transitional
20 endoplasmic reticulum ATPase met the criteria of an OF embryo-interacting protein or
21 peptide.

22 **Keywords:** Oviduct, Fallopian tube, peptidomics, proteomics, embryo.

23

24 **1. Introduction**

25 Cattle embryos develop up to the 8-16 cell or morula stage in contact with the oviduct fluid
26 (OF) [1]. However, very few studies did investigate the molecular interactions between
27 proteins present in the OF and developing embryos [2-4]. Extracellular vesicles isolated from
28 the bovine OF and added to the culture medium were reported to be taken up by developing
29 embryos and to improve the developmental rates and embryo quality in terms of cell number,
30 hatching rates, cryosurvival and metabolism [5-7]. Intact cell matrix-assisted laser
31 desorption/ionisation time-of-flight (MALDI-TOF) – mass spectrometry (MS) allows a direct
32 detection of intact proteins, peptides and lipids from whole cells, with a high sensitivity (from
33 femtomolar to picomolar concentrations) and over a relatively wide mass range (1-30 kDa)
34 [8]. Recently, MALDI-TOF MS applied on *in-vitro* produced embryos increased our
35 comprehension of the phospholipid composition of bovine blastocysts under different culture
36 conditions [9, 10]. Here we applied MALDI-TOF MS on intact single bovine embryos
37 fertilized *in vitro* and pre-exposed or not to post-ovulatory OF in order to detect OF proteins
38 and peptides possibly uptaken by the embryos. We hypothesized that the uptake of OF
39 proteins and peptides would increase over time the abundance of specific OF masses in the
40 MS profiles of OF-treated embryos compared to untreated controls.

41 **2. Materials and methods**

42 All reagents were purchased from Sigma-Merck (Saint-Louis, MO, USA) unless otherwise
43 stated. PMSG, hCG and PG-600 were obtained from MSD Animal Health (Brussels,
44 Belgium). Oviducts and both ovaries from adult cows were transported on ice from a local
45 slaughterhouse and processed for OF collection less than 2 h after death. Post-ovulatory OF
46 (based on the presence of a red, recently ovulated corpus luteum < 1 cm in diameter with cells
47 loosely organized, corresponding to approximately day 1-5 post-ovulation [11]) was collected
48 from the oviduct ipsilateral to the corpus luteum, as previously described [2]. The OF from 22

49 cows were pooled, assayed for protein concentration (69.5 mg/mL), divided into 15- μ L
50 aliquots and stored at $-80\text{ }^{\circ}\text{C}$ until use for incubation with embryos. In vitro embryo
51 production was carried out as previously described [2]. Briefly, in-vitro matured COCs (50
52 per well) and Percoll-washed spermatozoa from one Normande bull (Evolution, Noyal-sur-
53 Vilaine, France) at a final concentration of 1×10^6 spermatozoa/mL were co-incubated in
54 fertilization medium (Tyrode medium supplemented with 25 mM bicarbonate, 10 mM lactate,
55 1 mM pyruvate, 6 mg/mL fatty-acid free BSA, 10 μ g/mL heparin and 40 μ g/mL gentamycin)
56 for 22 h (Day 0). At Day 1, all presumptive zygotes were cultured in 25 μ L of synthetic
57 oviductal fluid (SOF, [12]) supplemented with 0.01 % of polyvinyl alcohol (SOF-PVA)
58 without any serum or protein supplementation, under mineral oil (M8410) at $38.8\text{ }^{\circ}\text{C}$ with 5
59 % CO_2 and 5 % O_2 . At Days 3 and 5, groups of 30-50 intact embryos of normal morphology
60 (spherical embryos with blastomeres uniform in size, color and density) at the 4–6 cell and
61 early morula (stage code 3 according to the IETS classification) stages, respectively, in 15 μ L
62 of SOF-PVA were randomly allocated to incubation in 15 μ L of OF (OF-treated embryos) or
63 in 15 μ L of SOF-PVA (controls), leading to a final volume of 30 μ L for 3 h or 6 h at $38.8\text{ }^{\circ}\text{C}$
64 in humidified atmosphere with 5 % CO_2 and 5 % O_2 . After incubation, 10-15 embryos per
65 group were washed three times in 500 μ L of 20 mM Tris-HCl buffer (pH 6.8) supplemented
66 with 8.9 % sucrose (Tris-sucrose) then stored in groups of 10-15 at $-80\text{ }^{\circ}\text{C}$.

67 For MALDI-TOF MS analysis, embryos were thawed on ice then individually dropped with a
68 minimal volume of Tris-sucrose buffer onto the MALDI plate using a videomicroscope before
69 addition of 0.5 μ L of saturated α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution
70 containing 50 % acetonitrile / 50 % H_2O in presence of 0.2 % trifluoroacetic acid (TFA). The
71 matrix/sample was allowed to evaporate at room temperature for 30 min before MS analysis.
72 In addition to embryos, one aliquot of the frozen-thawed OF used for co-incubation was
73 desalted and concentrated using Zip-Tip C_4 solid-phase extraction (Merck Millipore,

74 Massachusetts, USA) and analyzed by MALDI-TOF with the same matrix (1 μ L of OF + 1
75 μ L of matrix): 10 technical replicates were performed. All mass spectra were generated using
76 a MALDI-TOF mass spectrometer UltrafleXtreme (Bruker Daltonics, Bremen, Germany)
77 equipped with a Smartbeam laser at 2 kHz laser repetition controlled by flexControl 3.4
78 software (Bruker Daltonics, Bremen, Germany) and operating in positive ion mode and linear
79 MS mode. External calibration was performed using 1 μ L of a standard mixture of peptides
80 and proteins (1:1; v/v in CHCA matrix solution): Glu1-fibrinopeptide B, ACTH (fragment18-
81 39), insulin and ubiquitin, each at 1 pmol/ μ L, 2 pmol/ μ L cytochrome C, 4 pmol/ μ L
82 myoglobin and 8 pmol/ μ L trypsinogen. Spectral profiles were acquired in the mass range of
83 2,000 -25,000 m/z with 5000 shots per spectrum. All embryos from one IVF were analyzed
84 on the same MALDI plate and all analyses were made within two weeks. To increase mass
85 accuracy (final mass error < 0.05%), an internal calibration was applied to all spectra by
86 performing a lock mass correction using the m/z at 8,452 or 8,565 corresponding to the
87 ubiquitin protein (naturally present in embryos). In total, 140 4-6 cell embryos (3-h treatment:
88 29 treated, 29 controls; 6-h treatment: 40 treated, 42 controls) and 144 morulas (3-h treatment:
89 29 treated, 27 controls; 6-h treatment: 47 treated, 41 controls) originated from 3 or 4 IVF were
90 individually analyzed.

91 The data were analyzed using the MALDIquant package adapted for mass spectrometry data
92 [13] of the R software (version 3.6.2). The mass spectra were treated for baseline subtraction
93 as previously described for embryo lipidomics [9]. The normalization of intensity values was
94 performed using the total ion count method. Peaks were detected using a total average
95 spectrum and MAD (median absolute deviation) method with a signal:background noise > 2
96 and a frequency of detection in at least 10 % of all mass spectra. The repeatability of the MS
97 acquisitions was evaluated by calculating the coefficient of variation (CV) from the
98 normalized peak intensity values of the three technical replicates for each embryo (technical

99 variability). The average CV obtained was 27.9 %. The comparison between groups was
100 analyzed using the non-parametric Kruskal-Wallis test with the Benjamini-Hochberg
101 correction for multiple comparisons. The hierarchical clustering of differential masses was
102 performed using Spearman correlations and the gplot package (v 3.0.1.2) of FactoMineR (v
103 2.1). Comparison between treated and control embryos at each stage and incubation time was
104 analyzed using the non-parametric Mann-Whitney test. Masses were considered differentially
105 abundant between groups with a p-value < 0.05. Masses were considered as potential embryo-
106 interacting proteins originating in the OF if they met both following criteria: exclusive
107 detection in OF-treated embryos or abundance significantly increased in OF-treated embryos
108 compared to controls with a fold-change ≥ 2 in at least one stage \times incubation-time
109 combination; and identification by MALDI-TOF MS in the OF with a final mass error of
110 0.05%. Masses were annotated based on theoretical masses of $m/z \pm 0.05\%$ using a home
111 database generated from bovine granulosa cells and oocytes top-down proteomics [14] or
112 using top-down database compiled by the Consortium for Top-Down Proteomic (CTDP)
113 gateway (<https://www.topdownproteomics.org>).

114 **3. Results and discussion**

115 MALDI-TOF MS analysis of single bovine embryos evidenced up to 197 masses
116 corresponding to peptides and proteins in the range of 2 – 25 kDa (see Suppl. Table 1 for the
117 exhaustive list of masses quantified in embryos). The MS analysis of the pool of OF used for
118 embryo incubation evidenced a specific spectrum of 136 masses in the same mass range
119 (Suppl. Table 1). The hierarchical clustering of differential masses between groups evidenced
120 the embryonic stage (4-6 cell embryos *vs.* morula) as the most discriminating factor followed
121 by the treatment (OF-treated *vs.* control) and the time of incubation in OF (3 h *vs.* 6 h) (Figure
122 1). To our knowledge, this is the first use of MALDI-TOF MS to explore the peptidome and
123 proteome of bovine embryos. Our results show that the MS profiles of individual early

124 embryos could be discriminated according to their stage of development, the pre-exposure to
125 OF and time of exposure, showing the high sensitivity of this method to discriminate changes
126 in peptides and proteins from a low amount of material, although in a mass range less than 25
127 kDa. The stage-specific changes in embryonic MS profiles may rely on the variation in the
128 physico-chemical properties of the zona pellucida during embryo development [15] and on
129 the major activation of the embryonic genome, which occurs between the 8-cell and 16-cell
130 stages in the cattle embryo [16].

131 Exposure to OF had no apparent effect on the morphological quality of embryos at both stages
132 (data not shown). It was hypothesized that masses found overabundant or exclusively in OF-
133 treated embryos compared to controls may originate in the OF. No mass was specifically
134 detected in OF-treated embryos. By applying a minimal fold-change ratio of 2, a total of 16
135 differentially abundant masses were detected, among which 11 were overabundant in OF-
136 treated embryos: five exclusively in 4-6 cell embryos, four exclusively in morulas and two in
137 both stages (see the volcano plots of differentially abundant masses with annotations for 4-6
138 cell embryos and morulas in Figures 2 and 3, respectively; p-values and fold-change ratios in
139 Suppl. Table 1). Among the overabundant masses, one mass at m/z 8882.31 and annotated as
140 immediate early response 3-interacting protein 1 (IER3IP1, 9 kDa) or a peptide of transitional
141 endoplasmic reticulum ATPase (VCP, 89 kDa) was 3.4-fold more abundant in 4-6 cell
142 embryos compared to controls after 6 h of OF exposure and was also identified in the post-
143 ovulatory OF with a final mass error less than 0.05% (m/z 8878.14; Suppl. table 1).
144 Therefore, this mass of 8.9 kDa met the criteria to be considered as an embryo-interacting
145 protein or peptide originated in the OF. Previous data from our laboratory showed that protein
146 interactions in bovine 4-8 cell embryos were localized in the zona pellucida, perivitelline
147 space and into blastomeres [2], however, further experiments using specific immunostaining
148 would be necessary to precisely localize these interactions.

149 This result has to be taken with caution for several reasons. First, most masses found
150 overabundant in OF-treated embryos were not detected in the OF used for embryo co-
151 incubation and therefore did not meet the criteria for being considered as embryo-interacting
152 proteins. One hypothesis is that OF proteins/peptides internalized by embryos may have been
153 at levels under the detection threshold of MALDI-TOF MS as only 136 masses could be
154 quantified in the OF. Another hypothesis is that OF proteins with molecular weight higher
155 than 25 kDa were cleaved in smaller peptides by proteolysis at the time of their internalization
156 in embryonic cells and could thus not be detected in their native form in the OF due to the
157 mass range of MALDI-TOF detection. Furthermore, short incubation times (< 6 h) were
158 chosen to avoid the detection of proteins neosynthesized by the embryo, however, we cannot
159 exclude an increase in embryo synthesis of specific proteins due to OF exposure, leading to
160 the differential abundance of embryo-derived masses between groups. The transcriptional
161 activity and protein synthesis in embryos during short incubation with OF have not been
162 investigated so far. However, under basic culture conditions, a transcriptional activity has
163 been reported to occur in bovine 4-cell and 8-cell embryos [17]. Further studies are needed to
164 determine if masses found increased in abundance in OF-treated embryos are due to
165 endogenous embryo protein synthesis or to internalization from the OF with possible protein
166 cleavage.

167 In conclusion, the MALDI-TOF MS analysis of individual embryos pre-exposed to OF
168 allowed us to identify one possible protein/peptide originated from the oviduct and interacting
169 with the early bovine embryo. Further work is now needed to identify the roles of these
170 interactions.

171 **Author contributions:** MSD and VL conceived and designed the experiments; CB performed
172 in vitro experiments and proteomic analyses; APTG and DT contributed to the analysis of

173 proteomic data; VL, BG and PM provided guidance and resources; MSD administrated the
174 project; MSD and CB wrote the paper; all authors read and approved the manuscript.

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230

231

232 **Figure legends**

233 **Figure 1.** Hierarchical clustering of the differentially abundant masses ($p < 0.05$)
234 characterized between stages of development (4-6 cell embryos *vs.* morula), treatment (OF-
235 treated in green frames *vs.* controls in purple frames) and times of exposure to OF (3 h *vs.* 6
236 h). Each column represents the mean intensity value per group and each line corresponds to
237 one mass. For a given condition and mass, red lines represent higher abundance while blue
238 lines represent lower abundance compared with other conditions. The proximity between
239 conditions and peptide/protein profiles are shown by the hierarchical trees on the top and left,
240 respectively.

241 **Figure 2:** Volcano plot analysis of peptides and proteins quantified in 4-6 cell embryos after
242 3-h (A) and 6-h (B) exposure to post-ovulatory OF. In each plot, the horizontal and vertical
243 dotted lines demarcate the p -value at 0.05 and fold-change ratio ≤ 0.5 or ≥ 2 , respectively.
244 Dark blue points indicate significantly less abundant masses with OF-treated:control ratios \leq
245 0.5 while red points indicate overabundant masses with OF-treated:control ratios ≥ 2 . Gene
246 symbols are indicated for annotated masses. The red arrows indicates a parallel identification
247 in the OF with a final mass error less than 0.05%. ATP5IF1, ATPase inhibitor, mitochondrial;
248 CKS1B, cyclin-dependent kinase regulatory subunit 1; COX7A2P2, putative cytochrome c
249 oxidase subunit 7A3; FGB, fibrinogen beta chain; H2B1H, histone H2B type 1-H; H2BC4,
250 histone H2B type 1-C; HBA, hemoglobin subunit alpha; IE3IP1, immediate early response 3-
251 interacting protein 1; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4;
252 VCP, transitional endoplasmic reticulum ATPase.

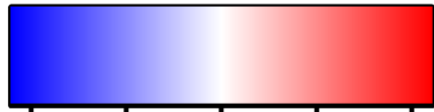
253 **Figure 3:** Volcano plot analysis of peptide/protein masses quantified in morulas after 3-h (A)
254 and 6-h (B) exposure to bovine post-ovulatory OF. In each plot, the horizontal and vertical
255 dotted lines demarcate the p -value at 0.05 and fold-change ratio ≤ 0.5 or ≥ 2 , respectively.

256 Dark blue points indicate significantly less abundant masses with OF-treated:control ratios \leq
257 0.5 while red points indicate overabundant masses with OF-treated:control ratios \geq 2. Gene
258 symbols are indicated for annotated masses. COL1A1, collagen alpha-1 chain; H2BC4,
259 histone H2B type 1-C; HMGN3, high mobility group nucleosome-binding domain-containing
260 protein 3; OGDH, oxoglutarate dehydrogenase; PTMA, Prothymosin alpha; TAGL2,
261 transgelin-2; RPL39, 60S ribosomal protein L39.

262 **Supplementary table 1.** List of peptidomic and proteomic masses (m/z) quantified in the
263 post-ovulatory oviduct fluid (OF) and in 4-6 cell embryos and morulas after incubation or not
264 (controls) in OF during 3 h or 6 h *in vitro*.

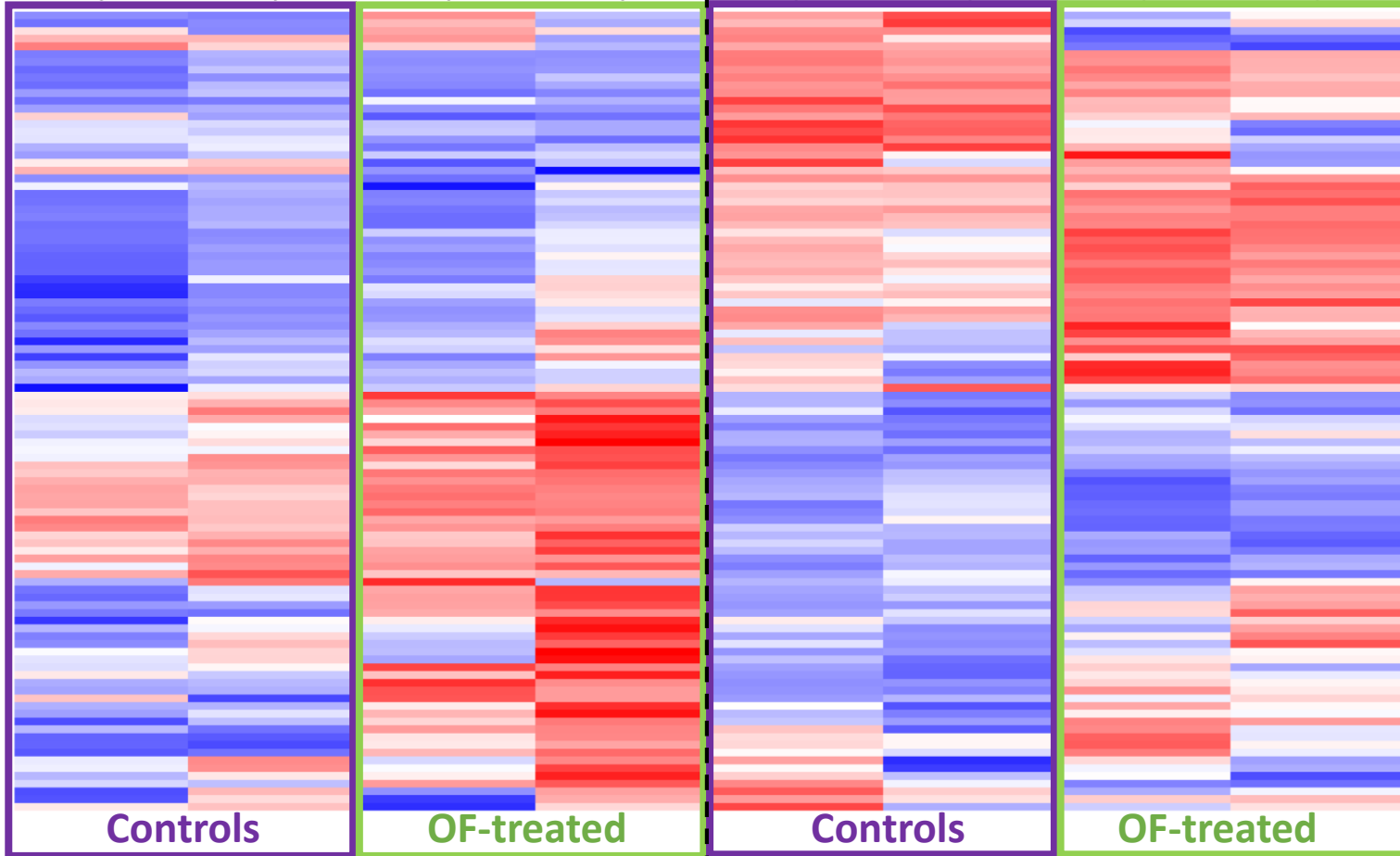
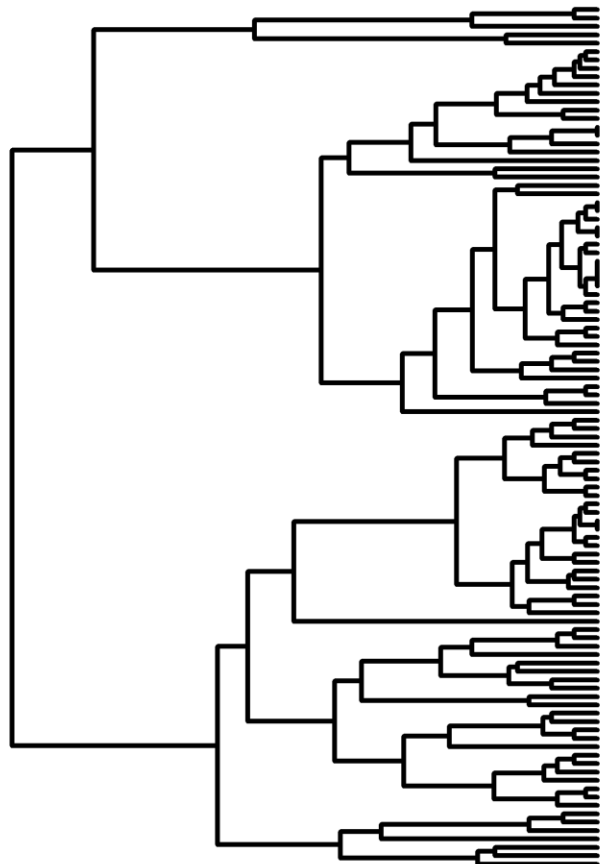
265

Color Key



-2 -1 0 1 2

Row Z-Score



Controls

OF-treated

Controls

OF-treated

3h

6h

3h

6h

3h

6h

3h

6h

Morulas

4-6 cell embryos

24696.95
15752.56
9539.08
10014.42
12552.95
10098.58
10721.52
13888.64
10411.84
4816.88
6355.56
5268.58
8992.34
10989.94
9025.56
8314.62
9929.8
7615.94
9957.4
12538.83
4525.41
6620.12
5943.46
9072.53
7782.34
4587.55
3767.82
6519.96
6533.49
5652.48
15369.78
15657.19
15601.85
12224.43
6116.51
15311.9
6867.66
7528.1
8890.27
8713.11
4973.91
5605.56
9164.95
13771.84
17854.23
6641.16
8599.67
8390
5137.6
11114.01
7653.49
5257.14

Masses

