

## Evaluation of polyvinyl alcohol for fatty acid supplementation in adipose tissue explant culture

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**ABSTRACT.** Cultures of adipose tissue explants are a valuable tool for studying the intracellular mechanisms involving hormones and nutrients. However, testing how fatty acids affect cells requires a carrier molecule; bovine serum albumin (BSA) has been used for this purpose. However, contaminants can alter the cellular response. Our objectives were to: 1) test BSA as a fatty acid carrier and 2) evaluate polyvinyl alcohol (PVA) as a replacement for BSA. Adipose tissue explants from nine pigs were cultured in medium 199 for 4, 12, 24, and 48 h, with the following treatments: control, PVA (100 mM PVA added) and PVA + pGH (100 mM PVA plus 0.1 mg/mL porcine growth hormone). After each culture period, explants were collected and assayed for lipogenesis. After 48 h in culture, explants were assayed for lipolysis. A preliminary study with different commercial sources and high concentrations showed that BSA affected lipogenic rates. On the other hand, there were no effects of PVA on lipid synthesis, while pGH (positive control)

reduced glucose incorporation into lipids ( $P < 0.01$ ) when compared to both control and PVA ( $P < 0.05$ ). There was no difference between control and PVA for lipolysis rates. However, pGH increased lipolysis when compared to control ( $P < 0.01$ ) and PVA ( $P < 0.05$ ). We demonstrated that BSA can alter lipogenesis, which precludes its use as a carrier molecule. On the other hand, addition of PVA had no effect on lipolysis or lipogenesis. We suggest the use of PVA instead of BSA for adding bioactive fatty acids to cultures of adipose tissue.

**Key words:** Polyvinyl alcohol, Cultures of adipose tissue, Bovine serum albumin, Bioactive fatty acids

## INTRODUCTION

Adipose tissue explant culture is an important tool for studying the intracellular mechanisms of hormones and nutrients, including fatty acids (FA). Tissue culture allows us to test several treatments simultaneously, quickly, and at a low cost. However, evaluation of the effects of FA on these cultures requires a carrier molecule. Bovine serum albumin (BSA) has been used for this purpose; however, some studies have shown that contaminants can affect the cellular response (Walton and Etherton, 1986) and can mask the effects of the FA (Tronstad et al., 2001). Different methods to complex FA with BSA have been published; these include differences in temperature, FA:BSA proportion, and incubation time (Mahoney et al., 1977; Park et al., 1997; Brown et al., 2001; Matitashvili et al., 2001; Evans et al., 2002).

The several methods for adipose tissue culture generally use cell lineages (e.g., 3T3-L1 preadipocyte line) or a primary culture system (e.g., explants or isolated cells). Each culture model has advantages and disadvantages, and the differences among models usually make it difficult to perform inter-study comparisons. Primary cultures of pig adipose tissue have been successfully used to evaluate the effect of hormones and nutrients on lipogenesis rates (Dunsha et al., 1992; Lanna et al., 1994; Etherton et al., 1995; Etherton and Bauman, 1998). In this species, lipid synthesis predominantly occurs in the adipose tissue, about 80% by *de novo* pathways. We evaluated polyvinyl alcohol (PVA) as a substitute of BSA for FA supplementation in adipose tissue cultures.

## MATERIAL AND METHODS

### Pig adipose tissue culture explants

Nine Landrace x Large White castrated male pigs with an average weight of 78 kg were humanely slaughtered and used as a source of adipose tissue. The pigs had been fed a 78% corn and 19% soybean meal diet, which had a 3.3% ether extract (percentages on a dry matter basis). Approximately 7% of the metabolizable energy came from lipids, and animals had free access to feed and water until slaughter. About 20 g of subcutaneous adipose tissue was removed postmortem from the upper leg (ham) of each pig and immediately placed in transport

buffer (25 mM HEPES, 0.15 M NaCl, 37°C, pH 7.4). In the laboratory, 50 to 100 mg sections (explants) of the intermediate layer of the subcutaneous adipose tissue were aseptically removed and placed into multi-well plates containing Medium 199 with Earle's salt, 0.1 g/L L-glutamine, 25 mM HEPES, and 25 mM sodium bicarbonate, supplemented with antibiotics and maintained at 37°C, pH 7.4 (Lanna et al., 1995). Three explants were used for each hormonal treatment for each pig. Control cultures were supplemented with 0.1 µg/mL insulin and 10 nM dexamethasone; PVA cultures received 100 µM PVA (Sigma Chemical, St. Louis, MO) and pGH cultures received 0.1 µg/mL pGH (porcine recombinant growth hormone - Reporcin®, Southern Cross Biotech) and 100 µM PVA. The cultures were incubated in 5% CO<sub>2</sub> at 37°C for 4, 12, 24, and 48 h. Some explants from each pig and treatment were collected and assayed for lipogenesis. After 48 h, the explants were immediately assayed for lipolysis.

### Lipogenesis assay

After incubation, the explants were transferred to scintillation vials containing 2 mL lipogenesis medium (1X Krebs Ringer buffer - 0.65 mM CaCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, and 0.5 µCi/mL glucose D-[<sup>14</sup>C(U)] - Amersham Biosciences; pH 7.4) and incubated for 2 h in 5% CO<sub>2</sub> at 37°C in a shaking water bath. This was done in triplicate for: a) basal lipogenesis - no insulin added and b) lipogenesis - with 1 µg/mL insulin. Subsequently, lipids were extracted from tissue explants, according to Folch et al. (1957), with some modifications. Explants were transferred to extraction vials containing 5 mL of a 1:2 methanol:chloroform mixture and incubated for 1 h at room temperature and 1 mL 1% NaCl was added. After 12 to 16 h of incubation, the lower phase was collected, transferred to scintillation vials, and 0.5 mL hexane and 5 mL scintillation liquid were added (Sigma Chemical). Radiation was measured in an automatic scintillator (Beckman Instruments - LS 5000 TD - Liquid Scintillation System), and the lipogenesis rate is expressed in nmols of glucose incorporated per gram of tissue in 2 h.

### Lipolysis assay

After 48-h incubation, explants were collected and incubated in 2 mL lipolysis medium (1X Krebs Ringer buffer - 0.65 mM CaCl<sub>2</sub>, 2.5 mM HEPES, 5.6 mM glucose, and 3% BSA, pH 7.4) for 2 h in 5% CO<sub>2</sub> (v/v) at 37°C in a shaking water bath. This was done in triplicate as follows: a) basal lipolysis - nothing added and b) lipolysis - 10 µM isoproterenol added. Samples of the medium were then collected for non-esterified fatty acid analysis (NEFA) by enzymatic colorimetric method, at 540 nm, using the NEFA C® kit (Wako Chemicals). Absorbance was measured in a microplate ELISA reader (Spectra Max Plus - Molecular Devices). Absorbance values were converted into concentration (µmol of oleic acid/L) based on the standard curve, and NEFA concentrations were corrected for explant weight (g) and are expressed in µmoles of oleic acid per gram of tissue produced for 2 h.

### Statistical analysis

Data were analyzed by a Mixed Procedures module (SAS, 1999). For the lipogenesis assay, an ANOVA analysis was made, considering repeated measures along time, a model involving fixed effects of treatment, stimulus, time, and interactions, and random effects on

animal in the treatment and animal in the treatment\*stimulus. Covariance was calculated to compensate for time differences - SP(POW) (time), according to Littell et al. (1996). ANOVA was used to compare treatments in the lipolysis assay, using a split plot model, in which treatments were applied to parcels and stimuli to sub-parcels. All means were compared by the Tukey test at 5% or at 5 and 10% of significance for lipogenesis and lipolysis, respectively.

## RESULTS AND DISCUSSION

Preliminary assays using BSA-fraction V, FA free in the culture medium showed a numeric variation in lipogenic response. The rate of glucose incorporation after 24 and 48 h of culture was higher in the medium containing BSA, insulin and dexamethasone in comparison with insulin and dexamethasone alone (control). Means were compared by the Student *t*-test at 5% of significance (Table 1 and Figure 1).

**Table 1.** Lipogenesis rate (nmol of glucose incorporated g tissue<sup>-1</sup> 2 h<sup>-1</sup>) in adipose tissue explants, 24 and 48 h of culture with different treatments.

	Treatments			
	BSA	Control	BSA	Control
	Baseline		Insulin	
Time				
24 h	2,592.7 <sup>a,A</sup>	1,477.6 <sup>a,A</sup>	2,428.0 <sup>a,A</sup>	2,100.6 <sup>a,A</sup>
48 h	1,912.9 <sup>a,A</sup>	870.2 <sup>a,A</sup>	2,513.2 <sup>a,A</sup>	1,109.9 <sup>a,B</sup>

Treatments: control (0.1 µg/mL insulin and 10 nM dexamethasone); BSA (0.1 µg/mL insulin, 10 nM dexamethasone and 100 µM bovine serum albumin).

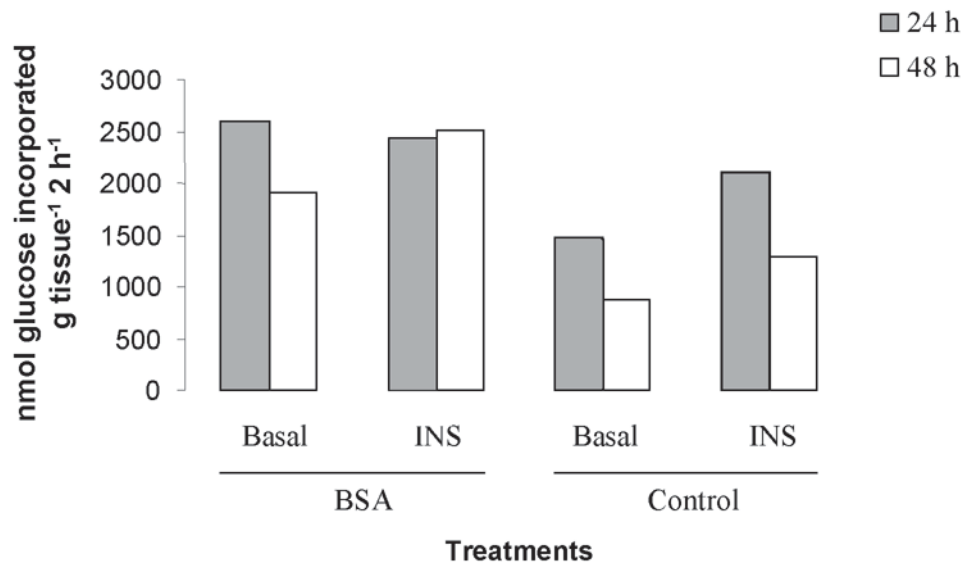
Comparison between baseline (no addition) and insulin (1 µg/mL insulin).

Lower case letters refer to row comparisons and capital letters to column comparisons.

Different letters indicate difference at  $P < 0.05$ .

Although no significant differences were detected (Table 1 and Figure 1), considerable variation (over 70%) in the basal lipogenesis rate was found in comparison with the control to BSA after 24- and 48-h incubation. This same trend was observed for lipogenesis stimulated by insulin, mainly after 48 h of culture, when the lipogenesis rate increased over 100% with BSA. Similar responses have also been observed (at a higher or lower level) for different concentrations, lots and commercial sources of BSA, even when dialysis-purified BSA was used (Spector, 1986). Possibly, BSA contains other lipogenesis-stimulating components, which could act both with or without a stimulus.

We found that high concentrations of BSA can alter the insulin stimulatory effect on lipogenesis in pig adipose tissue (Figure 1), as also found by Walton and Etherton (1986). They also observed differences between commercial BSAs, measured by their effects on lipogenesis in pig adipose tissue explants incubated for 2 h. These authors suggested that contaminants in commercial preparations include insulin-like growth factor, steroids and others. These findings led us to search for an alternative carrier molecule for adding FA to culture media; PVA was



**Figure 1.** Lipogenesis in adipose tissue explants after 24 and 48 h of culture. BSA (insulin, dexamethasone and 200  $\mu$ M bovine serum albumin) and control (insulin and dexamethasone). Results are reported in  $\mu$ mol of  $^{14}$ C-glucose incorporated per gram of tissue after 2-h incubation with no addition (baseline) or 1  $\mu$ g/mL insulin (INS).

tested as an option. When we examined the effect of PVA on  $^{14}$ C-glucose incorporation (Table 2), we found no significant effects in the interactions: treatment\*stimulus ( $P = 0.77$ ), treatment\*time ( $P = 0.28$ ), time\*stimulus ( $P = 0.79$ ), and time\*treatment\*stimulus ( $P = 0.85$ ). However, there were significant effects of treatment ( $P < 0.001$ ), time ( $P < 0.001$ ) and stimulus ( $P < 0.05$ ).

The rate of  $^{14}$ C-glucose incorporation into lipids was lower in the pGH treatment than for other treatments (Table 2). This reduction is consistent with several studies involving GH and demonstrates that the culture system was metabolically active and responded to hormone stimuli.

No significant differences in lipid synthesis rate were detected between the control and PVA treatments ( $P = 0.73$ ). This result corroborates our hypothesis that PVA does not alter lipid metabolism in primary cultures of adipose tissue explants. The inhibitory effect of pGH on lipogenesis was also observed when PVA was added to culture, demonstrating that response to pGH is unaltered by PVA. In addition, PVA had no effect on the lipid synthesis pattern during incubation. The lipolysis values (liberation of NEFA after 2-h incubation) were not affected by PVA (Table 3,  $P = 0.92$ ), but there were effects of treatment ( $P < 0.01$ ) and stimulus ( $P < 0.001$ ). Interaction between treatment and stimuli was not significant ( $P = 0.44$ ).

Absence of treatment\*stimulus interaction indicates that explants responded to treatment in a similar way, regardless of stimulus. For example, treatment with pGH induced increased NEFA release, both with and without isoproterenol ( $\beta$ -adrenergic). This is consistent with the ability of pGH to stimulate lipolysis and also confirmed that the system was able to respond to a lipolytic stimulus.

**Table 2.** Lipogenesis rates in adipose tissue explants after 4, 12, 24, and 48 h of culture with different treatments.

Time	Stimulus <sup>1</sup>	Treatments		
		Control	PVA	pGH
(nmol glucose incorporated g tissue <sup>-1</sup> 2 h <sup>-1</sup> )				
4 h	Baseline	7726.7	8272.5	4992.5
	Insulin	9373.1	1003	7163.2
12 h	Baseline	5923.4	5017.3	5003.7
	Insulin	7286.2	6352.8	6238.9
24 h	Baseline	5496.4	5389.0	3225.7
	Insulin	8698.8	6901.3	5753.6
48 h	Baseline	3347.9	2231.8	1660.3
	Insulin	4997.3	2911.4	1500.2
Lipogenesis <sup>2</sup>		6409.6 (1090.7)	5914.4 (1090.7)	3804.6 (1112.8)
		Control vs PVA ns	Control vs pGH **	PVA vs pGH *

Treatments: control (0.1 µg/mL insulin and 10 nM dexamethasone); PVA (100 µM polyvinyl alcohol) and pGH (0.1 µg/mL porcine growth hormone).

<sup>1</sup>Average between baseline (no addition) and stimulated (1 µg/mL insulin).

<sup>2</sup>Lipogenesis: means and their respective standard errors, in nmol of glucose incorporated per g of tissue after 2-h incubation, in adipose tissue explant cultures of nine animals.

Probability of difference between treatments (\*P < 0.05; \*\*P < 0.01; ns: P > 0.05).

**Table 3.** Lipolysis rates (liberation of fatty acids after 2-h incubation) in adipose tissue explants cultured for 48 h under different treatments.

Stimulus <sup>1</sup>	Treatments			Median ( $\bar{X}$ )
	Control	PVA	pGH	
(nmol NEFA g tissue <sup>-1</sup> 2 h <sup>-1</sup> )				
Baseline	1.75 (0.85)	1.85 (0.90)	3.65 (0.85)	2.41
Isoproterenol	7.29 (0.90)	4.73 (1.70)	9.05 (0.90)	7.02
		Control vs PVA ns	Control vs pGH **	PVA vs pGH *

Treatments: control (0.1 µg/mL insulin and 10 nM dexamethasone); PVA (100 µM polyvinyl alcohol) and pGH (0.1 µg/mL porcine growth hormone).

<sup>1</sup>Comparison between baseline (no addition) and stimulated (10 µM isoproterenol) lipolysis.

Lipolysis: means and their respective standard errors in µmol of non-esterified fatty acid analysis (NEFA) per g of tissue for 2-h incubation with stimulus, after 48-h culture with different treatments.

Probability of difference between treatments (\*P < 0.05; \*\*P > 0.05; ns: P > 0.10).



The results of the lipolysis assays were similar to those for lipogenesis and confirm that adding PVA to culture medium does not alter explant metabolism. This was observed even in long-term culture (48 h).

Along with the variability in the effect of albumin on *in vitro* lipid synthesis, the methodologies proposed in the literature are inconsistent regarding the formation of the FA:BSA complex. The methodology used by Park et al. (1997) differs from the one used by Mahoney et al. (1977) in relation to ideal temperature and period needed to form the complex. Matitashvili et al. (2001) tested a 3:1 FA:BSA ratio in mammary gland tissue of lactating cows, while Brown et al. (2001) and Evans et al. (2002) tested a 4:1 FA:BSA ratio in human vascular stroma cells and 3T3-L1 preadipocytes, respectively. In our study, we conducted preliminary tests for complex formation with 3:1 and 4:1 FA:BSA ratios, and we used various incubation times and temperatures. These differences in methodology could explain, at least in part, the varied and even contradictory responses that have been found in *in vivo* studies.

PVA has been compared with BSA in models of embryo culture in mice (Nowshari and Bren, 2000) and bovines (Bhuiyan et al., 2004; Orsi and Leese, 2004). According to Nowshari and Bren (2000), biological products, such as BSA, which have been routinely used in embryo culture *in vitro* could expose embryos to infectious agents, reinforcing the need to look for alternative products, such as PVA. PVA is a polymer with a molecular weight similar to albumin and its use is safer as it makes the culture medium better defined. With the increasing interest in bioactive lipid research, we conclude that this molecule can serve as an important tool for evaluating the effects of different lipids on tissue culture.

## CONCLUSIONS

PVA added to cultures of adipose tissue explants did not alter the patterns of lipogenesis or lipolysis both in control and growth-hormone-stimulated cultures. Moreover, cultures showed the same patterns of chronic responses to growth hormone and acute responses to insulin observed in *in vivo* and *in vitro* studies. In contrast, BSA stimulated lipogenesis, and BSA from different sources had variable and inconsistent effects on adipose tissue culture metabolism. Therefore, replacing BSA with PVA seems to be a suitable alternative for the evaluation of the effect of bioactive lipids on *in vitro* cultures, both functionally and to reduce cost.

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