The use of stirred suspension bioreactors as a novel method to enrich germ cells from prepubertal pig testis

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Spermatogonial stem cells (SSCs) are the foundation of male fertility. Their low abundance and the lack of reliable markers for SSCs are major road blocks in their isolation and use in germ line modification.

Differential adhesion of germ and somatic cells to tissue culture surfaces (differential plating) has been used to enrich undifferentiated spermatogonia, that include SSCs, from heterogeneous testicular samples on a large scale (Luo et al. 2006). However, this approach is very labor intensive which creates a need for a protocol that can yield significant enrichment of spermatogonia with less handling where scalability is possible. Suspension bioreactors have been routinely used to culture homogenous cell populations in large quantities, and they have advantages over static cultures in that they utilize standardized and controlled culture conditions in a scalable environment (Krawetz et al. 2011).

The objective of this study was to investigate if germ cells could be enriched in stirred suspension bioreactors based on the differential adhesion properties of testicular cells. We also investigated if stirred suspension cultures, followed by differential plating, could provide additional enrichment. Methods: testicular cells were harvested from pre-pubertal (10 week old) pigs by a two-step enzymatic protocol (Luo et al. 2006). At this age, spermatogonia and Sertoli cells are the only cell types present in the seminiferous tubule. Testicular cell suspensions were submitted to three different enrichment methods: stirred suspension culture (A), differential plating (B) and stirred suspension culture followed by differential plating (C), n = 3 replicates each. For enrichment method A: $5x10^6$ cells/ml were cultured in 100 ml stirred suspension bioreactors (NDS Technologies, Vineland, USA) as previously reported (Shafa et al., 2012) and agitated for 48 h at 100 r.p.m. in high-glucose DMEM (Invitrogen, Carlsbad, USA) supplemented with 50 IU/ml penicillin, 50 U/ml streptomycin and 5% FBS (Invitrogen). Every 24 h the cell suspension was filtered through a 40 μm mesh to remove large aggregates of somatic cells and such attain enrichment of germ cells. For method B: $7x10^6$ cells/ml cells were plated on 100 mm tissue culture dishes in 15 ml DMEM + 5% FBS supplemented with 50 U/ ml penicillin, 50 U/ ml streptomycin, and incubated at 37°C in 5% CO_2 in air. After 18 and 48 h, cells remaining in suspension and those slightly attached were removed by trypsinization (1:10 dilution of trypsin-EDTA), and plated on new dishes. For method C: cells enriched by stirred suspension for 48 h were plated on 100 mm tissue culture dishes for 24 h under the same conditions as treatment B. Cell recovery was recorded at all time points and cells were characterized by immunocytochemistry for Vasa and Vimentin to identify germ cells ($Vasa^+$, $Vimentin^-$) and somatic cells ($Vasa^-$, $Vimentin^+$), results were compared by ANOVA. Cells obtained from group A were also characterized by immunocytochemistry using antibodies against Gata4, P450c17, α smooth muscle actin and vimentin to identify Sertoli cells ($Gata4^+$, $Vasa^-$), Leydig cells ($P450c17^+$, $Vimentin^+$) and myoid cells (α $smooth\ muscle\ actin^+$, $Vimentin^+$) to investigate the effect of shear forces on testicular cells after 48 h, paired student t test was used to analyze these results.

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All three methods tested provided enrichment of spermatogonia in the final cell suspension when compared to the starting cell population. No significant difference was observed when comparing the fold of enrichment or recovery rates between methods A and B; in contrast, method C had significant enrichment ($p < 0.05$) and the total number of cells recovered was lower than methods A and B Table 1.

Table 1: Comparison of enrichment between methods

Method	*Time point*	*Vasa$^+$ (%)*	*Recovery (%)*	*Enrichment (fold)*
A	0 h	3.9 ± 1.7	100	
	48 h	19.4 ± 2.9	16.6 ± 1.2	5
B	0 h	6 ± 2.2	100	
	48 h	29.8 ± 10.2	25.6 ± 8.3	5
C	0 h	3.9 ± 1.7	100	
	72 h	35.6 ± 10	5.2 ± 4.4	9*

*$p < 0.05$

The characterization of the cell population recovered after 48 h in the bioreactor demonstrated that this method promotes Leydig and germ cell enrichment by eliminating cell aggregates formed by Sertoli cells, Table 2.

Table 2: Characterization of cell populations from the stirred suspension method

	Time in stirred suspension	
	0h	*48h*
Cell type	$\bar{X}$ *(%)*	$\bar{X}$ *(%)*
Myoid	4.4 ± 2.8	3.3 ± 0.2
Sertoli	72.8 ± 16.6	36.4 ± 0.8*
Leydig	5 ± 0.3	10.7 ± 2.6*
Spermatogonia	3.9 ± 1.7	19.4 ± 2.9*

*$p < 0.05$

These results indicate that stirred suspension culture is equally efficient as differential plating during 48 h to promote the enrichment of porcine germ cells from prepubertal testes. Stirred suspension culture followed by differential plating results in better germ cell enrichment, yet lower cell recovery. Therefore, enrichment of porcine germ cells in stirred suspension culture has advantages over differential plating, providing less handling, uniformly and reproducible results when large number of cells is needed. In conclusion, the enrichment by bioreactor offers a new and practical approach to attain germ cell enrichment from porcine prepubertal testis.

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