J Ayub Med Coll Abbottabad 2001; 13 (4)

CORNUCOPIA OF CULTURED SCHWANN CELLS: WHERE IS THE BEST SOURCE IN ALBINO RATS?

M. MIRAJULLAH, SHEN XINYA, LIU C AIDONG **, & Wang Yujiang****

Department of Anatomy, Ayub Medical College, Abbottabad, & ** Shanghai Medical University, PR China

Background: Schwann Cells (SC) arc multifunctional glia cells with diversified biological potentials. To study their biological activities, ample amount of cultured SC is a pre-requisite. Many sources have been exploited in the past for SC culture but unfortunately no attempt has been made to find the richest source. ***Methods:*** To make an attempt in this direction, we cultured SC under identical conditions from various neural sites in the neonate & young SD albino rats. Fresh, cryopreserved & pre-stimulated neural tissue- have been compared for total & % of SC/unit tissue from the combined data of living & immunostained cultures ***Results:*** Our data shows that the upper limb nerves have not only quantitative advantage ($2.35x10^4$ cells/rng tissue) but also have significant qualitative edge (P< .05- .01) over the remaining sources. It can be concluded from our data that upper limb nerves are the best available source for SC/unit tissue.

INTRODUCTION

Since the recognition of SC through the monumental work of Theodor Schwann[1] tremendous work has been done by numerous researchers, spread all over the corners of the world, to reveal the potential biological functions of these multifaceted glial cells. These highly versatile cells synthesize, secrete & express many neurotrophic, neurotropic, neurite promoting & growth factors'. They also play vital role in the development & organization of neural tissue & are indispensable for normal axonal insulation & functions. Their key contribution in the studies on nerve regeneration, demyelinating & neurological diseases is fast emerging.

Many sources; mammals, rodents, reptiles & avians; adult, young, neonate, fetal & embryonic; fresh, cryopreserved & autopsy material have been used in the past implying a galaxy of techniques to obtain copious amount of the cultured SC[2,3,4,5,6]. The struggle continues as we are still away from our goal of having ample amount of SC for our biological needs. Majority of the workers in the past have opted for sciatic nerves, most probably due to their easy removal [8,9,10,11,12]. An over-review of these studies indicate that little attention has been given to find & compare the rich sources of SC with quiet questionable & unsatisfactory data [6,7,913]. We made an attempt to find the richest neural source of cultured SC in SD albino rats.

MATERIALS & METHODS

We selected the following sources to find the richest source for cultured SC *in* the SD albino rats:
(a) fresh *peripheral* nerves of brachial plexus (F BPN)
(b) the classical source of fresh sciatic nerves (F SN)
(c) pre-degenerated gliomatic brachial plexus nerves (GBPN) & sciatic nerves (G SN)
(d) dorsal root ganglia-fresh (DRG)
(e) cryopreserved brachial plexus (CP BPN) & SN (CP SN)

For a, b, d & e neonate SD albino rats (PNI-3) were used. All the tissues were aseptically removed after decapitating the animals, series of washing in DW. 75% alcohol & 20 minutes' storage in the same. The tissues were collected in cold (l-4°C) DMEM (D 6280; Sigma Chemical Co, St Louis, MO, USA) & cleaned under dissecting microscope & then minced. The tissues were incubated in DMEM containing 10% FCS, 0.125% (w/v) trypsin (T 2271, type XII-s: Sigma Ch Co) & 0.6% (w/v) collagenase (C 9407, type XI: Sigma Ch Co) in hot water bath at 37°C for approximately 10 minutes with frequent shakings. Quickly full culturing medium (DMEM with J0% FCS) was added & gentle mechanical dissociation was carried out for few minutes. The tubes were then centrifuged at 1000rpm for 5 min, at the end of which viable cells could be seen settled down at the bottom. Half of the medium was replaced by fresh medium & the process repeated. In case of noticeable tissue debris, third wash was undertaken. Maximum amount of the medium was removed after final wash by repeated observation under phase contrast microscope to get rid of the remaining enzymes. The cells were diluted with fresh culture medium & total/viable cells count was carried out by trypan blue (0.4%) exclusion test[13]. The cells were cultured on poly-l-lysin (PLL: 100 µg/ml HBSS: P 6282; Sigma Ch Co) pre-coated & HBSS/DW washed round glass coverslips or culture bottles/dishes. The initial plating density was $4\text{-}8xl0^4$ cells/ml. The cells were kept in $C0_2$ incubator at 37°C, $5\%C0_2$, with full humid conditions for 2-3 Hrs after which the dishes were

flooded with culture medium. After 24Hrs the SC & fibroblasts (FB) were counted on the basis of morphological criteria[15,16,17] & somal-nucleus ratio[1]* along "y" **&** "x" axes under x250 phase contrast microscope (12x0.845mm area). Out of the total fields. 30 were selected randomly to get the total number of cells for that experiment. To further confirm this count, the cells were then immunocytochemically labeled (PAP or ABC method) with anti-LMN (L 9393: Sigma Ch Co) or anti-Sl00 (S 2644: Sigma Ch Co). The labeled cells were then counted under Nikon microscope as for the living cells.

For 'c' post suckling (PN20) SD rats were used. The animals were anaesthetized, area concerned was shaved & the nerves were exposed aseptically. The sciatic nerves were cut above the ischial tuberosities while the upper limb nerves were transected as high as possible near their trunks formation. The animals were returned to their cages & allowed to eat & drink ad libitum. After 7 days the animals were re-anaesthetized & the nerves were removed & processed for SC cultures like the fresh nerves with minor experimental adjustments. The optimal time of one week for Wallerian degeneration & post-transection (whereby in vivo enrichment & proliferation is allowed) was keeping in mind that very short periods don't permit the necessary length of time for cells proliferation while prolong denervation resulted into the blasting of FB.

For cryopreservation the denuded peripheral nerves of neonate animals were stored in the cryoprotectant fluid (20% glycerol v/v. 50% FCS. 30% DMEM with 2µg/ml of $Fe_2(SO_4)_3xH_2O$) into liquid nitrogen at -I96°C. The nerves were removed (after l-4months of storage) & quickly thawed in hot water at 37°C. They were washed with culture medium. The rest of the culture procedure was the same as mentioned above with appropriate changes in the digestion/enzymatic protocol. In some experiments the digested DRG cells were filtered through a sterilized nylon meshwork with pore size of about l00µ to reduce the neuron quantity & remove the debris. However, the procedure was found to give significant difference in counts & so was not included in the results.

RESULTS

Our short enzymatic treatment, meticulous mechanical dissociation, careful desheathment & use of PEL substratum[133] resulted into huge amount of SC. To make our cells count more reliable & perfect we not only used the combined morphological & nuclear-soma ratio criteria[18] but kept any cell with doubtful characteristic out of the data analysis.

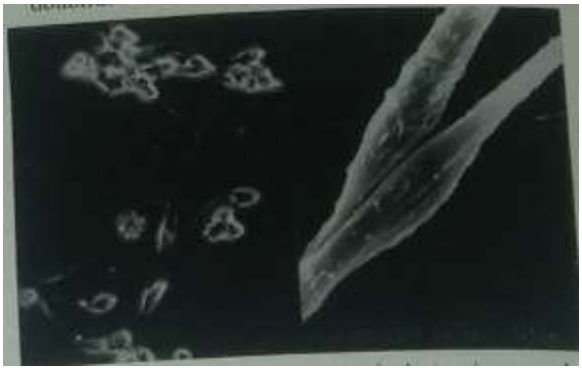

Fig-1: Phase contrast & SEIM photomicrograph of dissociated DRG & CP SN showing pleomorphic FB, phase bright SC, rounded dormant neurons. OM xl00 & x6K

The birefringence & nuclear refractivity of SC was not only a characteristic feature under the phase contrast but also under the SEM (Fig: I)

Lack of these qualities & relatively strong pasting made the FB nucleus prominent. The single sausage/oblong nucleus of SC usually contained 1-3 nucleoli & more than 50% of the somal area. In contrast the pleomorphic, non-refractive FB had more rounded nuclei studded with 2-6 nucleoli.

Table-1 gives the comparative data of cells count for various tissues based on 24-36 Hrs cultures without any antimitotic drug treatment. The cells count is based on randomly selected fields (see M 'M) & calculations for the whole area. The data average was calculated from the combined morphological & immunostained cells. It can be assessed at a glance from the table that as regards cells/mg tissue or cells/animal & SC%, the F BPN have significantly high cellular counts than the remaining sources ($P<0.05$ to 0.001). The 1.48% & 10.58% loss of total cells on cryopreservation in F BPN & F SN respectively (based on total average) can't be apprehended exactly. Possible reasons may be the different, at response(s) of the two sub-populations & the architectural difference in the nerves used.

Various techniques have been reported for separation of neuronal & non-neuronal cells in the past in mix cell population. In our study the SC survival dominated due to favorable culture conditions. Neurons were frequently seen at the initial stages of plating but didn't survive long enough due to inhospitable conditions. Table-2 gives the average weights of the cleaned, stripped & wet tissues from different sources. Fig II & III are representative figures from various tissues.

Table-1: Showing various cell counts / mg tissue

	FBPN	F SN	CP BPN	CP SN	GBPN	G SN	F DRG
	A	B	C	D	E	F	G
Total cells	27173 ±1755	21304±1474	26771 ±1091	19051 ±737	1584 ±88	1617 ± 74	14605± 1135
SC	23508 ± l586	17861 ± 1424	21962 ± 949	15585 ± 675	1199 ± 64	1241 ±72	11784 ± 1083
FB	3656± 186	3442 ± 230	4809 ± 699	3267± 135	385 ± 40	375 ± 57	2812±140
SC %	86 44 ± 00.34	82.13 ± 01 78	82 13 ±02 35	81.77 ± 00.99	75.77 ±01 75	76 61 ±03.13	77.86 ±02 98

(a) *P values for total cell counts are as follows: A* vs *B, D. E. F.G(P<0.01). A vs C (NS).* ***((b)*** *P values for SC are: A vs B (P <005). A vs D to G (P < 0 01). A vs C (NS),* ***(c)*** *P value for IB are as follows A Vs E. F G (P 0.001). A vs. B. C (NS),* ***(d)*** *P values for SC% are as: A vs B (P< 0 05).* . 1 *vs I). E. h (P - 0 01).* A ***vs*** *C(NS).* ***NS****: Non Significant*

Table-2: Showing average weights of tissue from various sources.

F/C BPN	F/CSN	G BPN	G SN	F DRG
A	B	C	D	E
2 62 ±	3.18 ±	22.61 ±	27 92 ±	1 43 ±
0.12	0.09	1.02	0.82	0.21
5	5	5	5	5

(a) The weight (mg/animal) given are that of fresh, wet and stripped tissue., (b) No. of animals in each experiment were 8 (fresh) & 5 (glioma) on average, (c) Figures in parenthesis arc number of experiments, (d) In case of DRG minimum of 60 ganglia (minimum of 3 animals for each experiment) were obtained from one animal.

Fig II: 24Hrs phase contrast photomicrograph of glioma dissociated cells showing many bipolar & some rounded SC along with debri. OMx200.

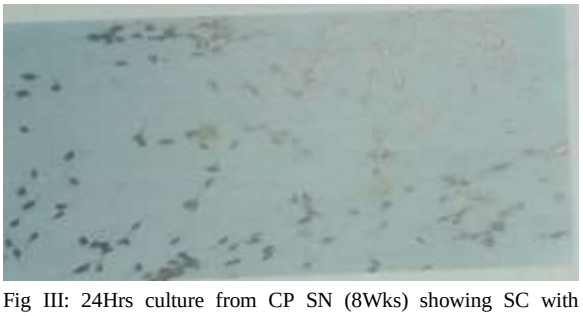

Fig III: 24Hrs culture from CP SN (8Wks) showing SC with different morphologies & unstained FB (LMN: ABC method). OMx100

DISCUSSION

Main objective of our study was to find the richest source of SC, in SD albino rats, by comparing SC/unit tissue processed under identical conditions without cytotoxic killings, mitogens stimulation or other drugs manipulations. As regards number of SC & SC% per mg tissue, our data clearly indicates the 'dominancy' of F BPN over the other sources. We used the protocol of less concentrations of trypsin & more collagenase producing not only mild cum faster digestion leaving no tissue fragment behind but also maintaining excellent cellular viability (approx.: 94%). The low concentration of trypsin would also certainly nave contributed to the increase in number of SC by minimizing damage to axolemmal mitogen(s)[15,19,20]. Utility of the superior PLL substratum yielded optimal plating efficiency in the presence of minimum quantity of culture medium.

A wide range of variations in cell counts from various tissues have been reported in the past[7,10,13,19,20]. Going through the literature it surmised us that the BPN have been scarcely used as culture source[17,21]. Both of these studies lack comparative quantitative data. Wrathall et al[17] did use ulnar nerves of adult cats but reported no difference with SN without giving any comparative data. Their count (2- 5x10^7/33 cats) can't be compared with our data for obvious reasons of species difference, use of a single nerve, partial digestion, exhaustive & vigorous experimental protocol. Their low (~16%) SC in the first 24Hrs & a sudden Para jump to 70% in 48-96Hrs indicates either the differential cellular attachment or the substratum factor. The reasons behind our high SC% may be the combined identification criteria making the counting escape difficult for the "so called" multipolar FB & the use of superior substratum.

The enviable high count (l-5x!0^4) reported by Wood[7] for fetal rat DRG can be attributed to the use of mitogens, age factor & prolong culture period[22]. Scarpini et al[19] have reported 1- 1.5xl0^{6}cells/SN for human fetuses. Employing the same protocol these workers have achieved much lesser counts for adult rat SN. Rutkowski et al[2,3] could get only 8-24x10^2

cells/mg of adult human sural nerve which was increased by x25 after 14 days of treatment with cholera toxin[12,24,25] Fields & McMenamin[26] could achieve only 2.5-0.5x10^4 SC from adult rat SN. Morrissey et al[27] have given SC' counts for adult human phrenic & rat SN ranging from 10^2 to l.35xl0^5. Boyer et al have reported 5.1x10^4 & 2.6x10^4 cells/mg from human Paediatric & adult autopsy material after cultures in the presence of mitogen for l-2wks. Despite the close similarities between our results & the total cells count or SC % reported by these workers, comparison can't be made for obvious reasons of species/age difference, tissue bulk, differences in experimental protocol, culture duration & use of mitogens. The SC exposed to mitogen will not reflect the original cells count. The data of Brockes et at[2"] is in partial agreement with our findings. While their 85% purity is slightly less than that of ours, their high count seems to be due to late counts or tissue quantities. Rattier et al[25] manipulated purification of 95-99% can't be compared with ours because we made no attempt to purify our cultures. Mason et al"' 24Hrs cultures of neonatal rat SN contained mostly broken cells & tissue fragments with some bipolar SC. It might be the result of prolong digestion, severe mechanical treatment & bypassing the vital washing steps. While their AraC treated counts are less than ours, their untreated higher counts may be the result of difference in counting time.

Although mix cell population of peripheral nerves can be stimulated & purified[6,7,11,15,23, 30], attempt to find a source offering large quantities of enriched SC would offer unique advantages. Our experimental protocol offers an unprecedented cornucopia of SC from BPN. It can save the highly expensive mitogens & the risk of transformations with copious virgin SC in hand. These cells can be used for various biological studies without any fear of cytotoxicity or altered biological activities & can be expanded at the hour of need.

Regarding the reason(s) for significantly higher SC counts & SC% from BPN, it may be attributed to difference in the developmental stage, functional background, axonal type & population.

REFERENCES

1. Schwann T. Mikroskopische unlersuch-ungen uber die uebereinsfimmung in der struktur und dem wachstun der here und p flanzen. Sander. Berlin. 1839
2. Askanas V. Engel WK, Dalakas MC. Lawrence JV, & Carter LS Human SC m (issue culture Histochemical & ultrastructural studies Arch Neurol. 1980; 37 329-37
3. Bhattacharyya A, Bracken bury R. & Partner N Neuron-SC signals are conserved across species: purification and characterization of embryonic chicken SC. J Neurosci. 1993. 35. M3
4. Dupm I: SC differentiation in clonal cultures of the neural crest. as evidenced by the anti-SC myelin pmlem mAb PNAS USA 1990; 87; 1119-23.
5. Eccleston PA. Mirsky R &. Jessen KR lypc-1 collagen preparations inhibit DNA synthesis in glial cells of the PNS Exp Cell Res. 1989. 182 1-173-85
6. Moretto (i. Kim SU, Shut DH. Pleasure DC. and Rizzuto N Long term cultures of human adult SC isolated front mil tipsy materials Acta Neurop tol. 1984. 64 15-21.
7. Wood 'W Separation of functional SC and neurons from normal peripheral nerve tissue Brain Res. 1976; 115. 361-75
8. Gue'nard V. Kleitman N. Morrissey TK. Bunge RP. and Aebischer P Syngeneic SC derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration J Neurosci 1992; 12(9) 3310-20
9. Maeda T. Mackinnon SE. Best 1J cl al Regeneration across "stepping Stone" nerve grads. Brain Res. 1993; 618(2) 196-202
10. Oda Y, Okada Y, Katsuda S, Ikeda K. & Nakanishi I A simple method for the SC preparation from newborn rat SN .1 Neurosci Methods! 1989. 28 163-9
11. Porter S, Glaser I. & Bunge RP Release of autocrine growth actor by primary and immortalized SC Proc Natl Acad Sci USA. 1987. 84 7768-72
12. Raft MC, Hornhy-Smiih A. & Brockes JP cAMP as a mitogenic for cultured rat SC. Nature. 198": 273 672-3
13. Brockes. IP. Fryxell KJ & lemke Oh Studies on SC culture & morphology J Exp Biol. 1981.95 215-30
14. Silam V Pizzuti A. Strada D cl al. Human neuronal cell viability demonstrated in culture after preservation Brain Res. 1988. 437 169- 74.
15. Salzer JL. & Bunge RP. Studies of SC proliferation. (luring development, Wallerian degeneration. direct injury J Cell Biol. 1980; 84 739-52.
16. Murry MR, &. Stout AP Characteristics of human SC in vitro Anat Rec. 1910: 84: 29543.
17. Wrathall JR, Rigamonti DO. & Kao CC Cultures enriched in Schwann-like cells from nerve segment of the adult eat Brain Res. 1981; 224:218-23.
18. Mirajullah M Studies on SC cultures; Best source, substrate dependent morphological characteristics & proliferation in SD albino rats. PhD thesis. Shanghai Medical University. 1995
19. Scarpini E, Kreider BQ, Lisak RP et al. Culture of human SC isolated from fetal nerves. Brain Res, 1988; 440: 261-6.
20. Scarpini K, Kreider BQ Lisak RP. & Pleasure PH Establishment of SC cultures from adult rat peripheral nerves. Exp Neurol. 1988. 102 167-76.
21. 21 In no M, & Suzuki K Isolation and morphologic characterization in vitro of SC' of the Twitcher mouse. Lab Invest.1990; 62. 704-12.
22. Mason PW. Attema BL, & DeVries GH Isolation and characterization of neonatal SC from cryopreserved rat SN. J Neurosci Res. 1992; 31. 731-44.
23. Rutkowski L. Tannekoon GI. & McGillheaddy.JE; Selective culture of mitotically active human SC's from adult sural nerves Ann Neurol, 1992; 31(6): 580-6
24. Raff MC. Abney I. Brokes JP. & Hornby-Smith A SC growth factors. Cell, 1978, 15: 813-22.
25. Rainer N, Glaser L. and Bunge RP. PC-12 as a source of neurite- derived cell surface mitogen, which stimulates SC division. J Cell Biol. 150-5
26. fields Kl. & McMenammP SC cultured from adult rats contain a cytoskeletal protein related to astrocyte filaments Dev Brain Res. 20 259-69.
27. Morrissey TK, Kleitman N, Goddard & M, Aebischer P SC differentiation in vitro Differences between cells from adult rat and human nerves. Soc Neurosci Abstr, 1991; 17 376.
28. Boyer PJ. Rutkowski L, Tuite G el al. Isolation of human SC from pediatric dorsal nerve roots from dorsal rhizotomy A adult SN from autopsy Soc Neurosci Absf.,1992; 18:1490
29. Brockes JP. Fields Kl. & Raff MC A surface antigenic market for rat SC Nature (London), 1977,266:364-66.
30. Kim SU. Yong VW, Watabe K. & Shin DH Human fetal SC in culture: Phenotypic expression & proliferative capability. J Neurosci Res, 1989; 22: 50-9