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Ankyrin inhibits binding of erythrocyte spectrin to phospholipid vesicles

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Abstract

The studies on binding of erythrocyte spectrin to frozen and thawed phospholipid liposomes and its inhibition by ankyrin were performed. It was found that ankyrin inhibited up to 60% binding of spectrin by phosphatidylethanolamine/phosphatidylcholine vesicles. It was able to dissociate up to 40% of spectrin from this complex. Ankyrin inhibition of binding of phosphatidylserine/phosphatidylcholine vesicles by spectrin, although much lower, was also observed.

Key words: Membrane skeleton; Ankyrin; Spectrin; Phosphatidylethanolamine; Phosphatidylserine

1. Introduction

The spectrin-based erythrocyte membrane skeleton is believed to play an essential role in maintaining erythrocyte shape, integrity and elasticity (for a review see, e.g., Steck [1]). The results of electron microscopic observations of spread red blood cell membrane skeleton [2,3] provided details of its fine structure. A membrane skeletal meshwork is formed by long (maximal length ~200 nm) spectrin tetramers joined at their ends by junctional complexes consisting of a single (37 nm, 13 actin subunits) actin filament [2] and other proteins; protein 4.1 and adducin as well as dematin (protein 4.9) [3]. In the observed images of the spread cytoskeletons spectrin tetramers are either bare or associated with one or a pair of distinct globules 9–12 nm in diameter that were suggested to be ankyrin [4–6] or ankyrin/band 3 complexes [2,7]. Ankyrin was identified as a high affinity spectrin binding protein in the erythrocyte membrane [5,8–10]. Ankyrins constitute a family of proteins observed in red blood cell membrane as bands 2.1–2.6 [5] of which the major component is band 2.1 (M_r 206 kDa). The primary structure of ankyrin has recently been determined and the ankyrin gene has been assigned to human chromosome 8 [11,12]. Isolated ankyrin binds spectrin and also the cytoplasmic domain of the anion transporter [13,14]. Apart from the high affinity binding site the presence of lower affinity receptor(s) for spectrin that appear to be aminophospholipids is suggested, since spectrin was proved to interact directly with the lipid domain in either natural membranes [15,16] or in model systems [17,18]. Despite that this type of interaction of spectrin has been demonstrated for more than 20 years, its physiological significance remains unknown. A question which we address in this communication concerns the interrelationship between these two types of receptors (i.e., ankyrin or aminophospholipids) for spectrin on the cytoplasmic surface of erythrocyte membrane. Isolated ankyrin inhibited binding of spectrin by phosphatidylethanolamine/phosphatidylcholine (PE/PC) vesicles. It was also able to partially dissociate spectrin from PE/PC vesicle–spectrin complex. The inhibition of binding of phosphatidylserine/phosphatidylcholine vesicles by ankyrin, although lower, was also observed. The data indicate that phospholipids (particularly phosphatidylethanolamine) may, at least in part, occupy the same binding site as ankyrin.

2. Materials and methods

Bovine erythrocyte spectrin dimer was purified by the extraction of red blood cell ghosts with low ionic

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Abbreviations: BSA, bovine serum albumin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine.

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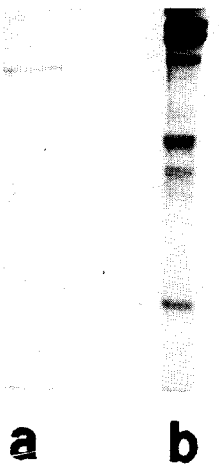

Fig. 1. SDS-polyacrylamide gel electrophoresis of ankyrin preparation. (A) Ankyrin purified by DEAE-Sephacel ion exchange chromatography followed by Sephacryl S-200 gel filtration. (B) Erythrocyte ghosts. Details in Section 2.

strength buffer at 37°C as was described previously [16,19] with small modifications described by Michalak et al. [20]. Erythrocyte membrane ankyrin was purified essentially according to Hall and Bennett [21], except that chromatography was carried out on 40 ml DEAE-Sephacel (Sigma) column using a linear gradient (0.2–0.5 M) of NaCl. During this and subsequent steps nonionic detergent was omitted from the buffers. Protein peak contained only ankyrin bands (mainly 2.1) as tested by SDS-(5%)-polyacrylamide gel electrophoresis [22]. In order to avoid the presence of traces of spectrin, ankyrin was additionally purified by gel filtration on Sephacryl S-200 column (1.5 × 35 cm) equilibrated with 0.6 M KI in 10 mM Na_2HPO_4, 1 mM EDTA, 1 mM DTT (pH 7.4). The SDS-(5%)-polyacrylamide gel electrophoresis pattern of major protein peak is shown in Fig. 1. Purified according to this protocol, ankyrin contained neither spectrin nor spectrin fragments, tested by immunoblotting with the use of anti-red blood cell spectrin antibodies (raised in rabbit) reacting strongly with β-subunit of spectrin (results not shown). Frozen and thawed (FAT) liposomes were prepared according to Hope et al. [23] using 20% dextran T-40 in the 'test buffer' [18] containing: 5 mM MES, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM DTT, 1 mM NaN_3 (pH 6.0 or 7.6) (at pH 7.6, MES was substituted with 5 mM Tris). The liposome suspension was diluted 10-fold with the test buffer (without dextran) and centrifuged at 30 000 × g to remove small vesicles and to wash off untrapped dextran. Spectrin and ankyrin were dialysed against the test buffer. In tests carried out according to Mombers [18], 200 μl of the mixture containing liposomes (500 μg phospholipid) and isolated spectrin (final concentration: 30–350 nM) was incubated at 20°C. After 30 min the incubation mixture was overlayered on 300 μl cushion of 0.5% dextran in the 'test buffer' and centrifuged at 30 000 × g for 6 min. Protein in the liposome pellet was assayed after addition of SDS to the final concentration of 0.1% according to the method of Dulley and Grieve [24] using purified spectrin as a standard. The data represent averaged values of triplicate experiments. The differences between the individual measurements did not exceed 10%.

In the inhibition experiments 400 μl of the incubation mixture contained: liposomes (1 mg phospholipid, final concentration $3.3 \cdot 10^{-3}$ M), 150 nM purified spectrin dimer (30 μg/sample) and indicated ankyrin concentrations. After a 30 min incubation at room temperature (20°C) the sample was overlayered on the 600 μl cushion of 0.5% dextran in the test buffer and centrifuged at 30 000 × g for 6 min. The liposome pellets were analyzed electrophoretically in SDS-polyacrylamide gel [22]. Spectrin bands from Coomassie blue-stained gels (stained overnight with 0.01% Coomassie blue R-250 in 5% acetic acid in 10% ethanol and destained with 5% acetic acid in 10% ethanol) were cut out and extracted with dimethylsulfoxide and the absorbance at 595 nm was measured versus the 'background' gel slice. Standard curve using pure spectrin was prepared. Each time, a standard sample of spectrin was run in the gel to make the necessary corrections. The data represent averaged values of 5–6 independent experiments. In the absence of ankyrin in the conditions of the incubation, 20–25% of added spectrin was bound by PE/PC and ~ 20% by PS/PC vesicles.

3. Results and discussion

In Figs. 2 and 3 the binding isotherms of spectrin to FAT-liposomes prepared from the mixture of PE and PS with PC at two pH values (6.0 and 7.6) are shown. Similar binding curves were obtained for the vesicles prepared from PC (data not shown). Nonlinear regression analysis performed with the use of computer program EZ-Fit (by F.W. Perrella, E.I. DuPont de Nemours, Glenolden laboratory, Glenolden, PA, received from Netserv, EMBL, Heidelberg) allowed to obtain K_d (dissociation constant) and B_{max} (maximal binding capacity) values shown in Table 1. The obtained K_d values are in the nanomolar range and are in good agreement with the data previously obtained by others [18,25]. Presented in the same Table, B_{max} values correspond to 1200 (PE/PC at pH 7.6) to 3600 (PC at pH 6.0) molecules of phospholipid bound per single molecule of spectrin dimer. These data are lower when compared to these of others [18,25], which might reflect the difference in the way of liposome preparation.

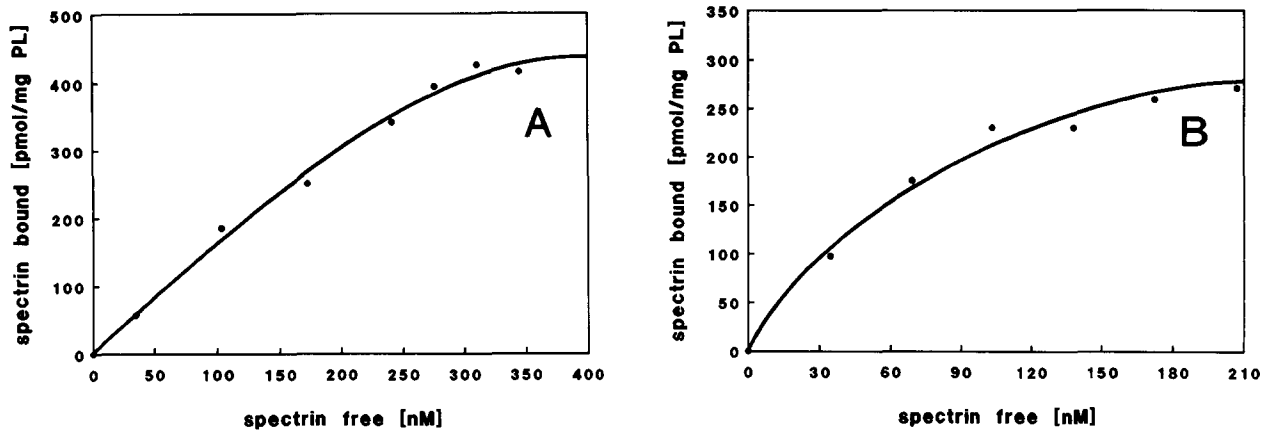

Fig. 2. Binding of increasing concentrations of isolated erythrocyte spectrin by phosphatidylethanolamine/phosphatidylcholine (60:40) FAT-liposomes. Binding assay performed at pH 7.6 (A) and at pH 6.0 (B). The average values of triplicate experiments are presented. The differences between individual measurements at particular spectrin concentration did not exceed 10%. Other details in Section 2.

For the initial experiments on ankyrin inhibition of phospholipid binding by spectrin, the liposomes prepared from the PE/PC (60:40) mixture were chosen. As is shown in Fig. 4A and B, isolated ankyrin is able to inhibit binding of purified spectrin to phosphatidylethanolamine-containing liposomes. However, not all binding was reduced. About 40% of binding was insensitive to inhibition by ankyrin. The effect of ankyrin on PE/PC vesicle binding at pH 7.6 (Fig. 4A) was very similar to the one at pH 6.0 (Fig. 4B). The effect of ankyrin does not seem to be nonspecific, since bovine serum albumin, which is known to bind many hydrophobic compounds at molar concentrations similar to the concentrations of ankyrin used in the above experiments, did not displace PE/PC vesicles from spectrin (Fig. 4C). Moreover, BSA bound to the vesicles, which was very well visible on the electrophoretogram of the pellets after the binding assay; they contained spectrin as well as BSA bands (not shown). It should be noted that BSA binds in a saturable manner to the vesicles but probably with much lower affinity, since the amount of spectrin bound remained unchanged. In contrast, ankyrin bands were not present in the Coomassie-stained gels resulting from electrophoresis of the liposome pellets, indicating, at least at these concentrations, an absence of ternary complex phospholipid-spectrin-ankyrin and also an absence of direct binding of ankyrin to liposomes. In Fig. 4D the Dixon [26] plot of the data resulting from the experiments carried out with the use of two different concentrations of spectrin (125 and 225 nM) may suggest a competitive type of inhibition with a K_i of 103 nM that is in good agreement with a K_d for ankyrin–spectrin complex (2–50 nM) [13,21]. It should be noted that, if both sets of data were fitted to the straight line (Fig. 4D) the following respective coefficients were found: a_0: 0.0764, 0.1916; a_1: 0.000397, 0.001515; and correlation coefficients: 0.939 and 0.951. The experiments presented in Fig. 5 indicate that when ankyrin was added after preincubation of spectrin with phosphatidylethanolamine-containing liposomes, the complex was dissociated. The final effect was smaller (30–40%),

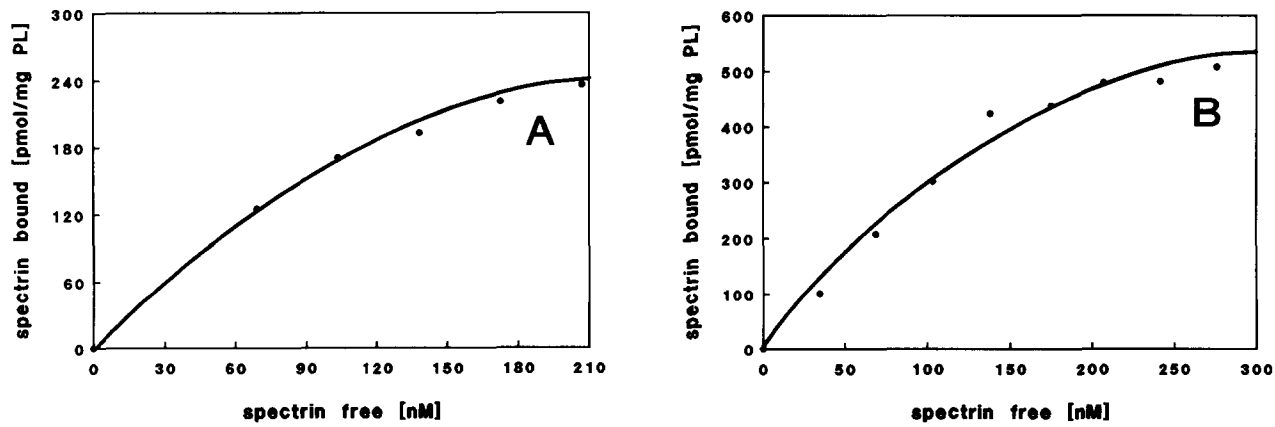

Fig. 3. Binding of increasing concentrations of isolated erythrocyte spectrin by phosphatidylserine/phosphatidylcholine (60:40) FAT-liposomes. Binding assay performed at pH 7.6 (A) and at pH 6.0 (B). Details as in Fig. 2.

Table 1
Dissociation constants and maximal binding capacities for purified bovine erythrocyte spectrin–FAT-liposomes interactions

	FAT-liposomes prepared from					
	PC		PE/PC (60:40)		PS/PC (60:40)	
	pH 6.0	pH 7.6	pH 6.0	pH 7.6	pH 6.0	pH 7.6
$K_d \pm$ S.E. (nM)	111 ± 17	99 ± 13	89 ± 14	577 ± 132	206 ± 39	146 ± 15
$B_{max} \pm$ S.E. (pmol/mg phospholipid)	370 ± 26	613 ± 35	405 ± 27	1162 ± 183	938 ± 95	403 ± 22

FAT-liposomes were prepared from PC, PE/PC (60:40) and PS/PC (60:40) The data were obtained with the use of computer program EZ-FIT by F.W. Perrella. Experimental details as in Figs. 2 and 3 and in Section 2.

indicating a possibility of the existence of several different binding sites. Similar to the case of the inhibition of binding of phosphatidylethanolamine-containing vesicles, there were no essential differences of the effect of ankyrin on the pre-formed complex PE/PC vesicle–spectrin at pH 7.6 and at pH 6.0 (Fig. 5).

Since there are several reports suggesting an apparent specificity of spectrin towards PS-containing liposomes (e.g., [17,18]) the effect of ankyrin on binding of spectrin by PS/PC (60:40) liposomes was also tested. An inhibitory effect of ankyrin on PS/PC vesicle binding by spectrin (Fig. 6) was much smaller (maximally ~ 20%). The effect of ankyrin on these liposomes binding seems to be pH dependent, being better pronounced at pH 7.6 than at pH 6.0 (Fig. 6).

To our knowledge this is the first demonstration that the binding sites for ankyrin and phospholipids (mainly phosphatidylethanolamine) may be at least in part overlapping. Why was this type of binding site not observed by Bennett and Branton [27] in their study on spectrin–spectrin-depleted inside-out vesicles interaction? First of all the K_d of spectrin-PE/PC interaction

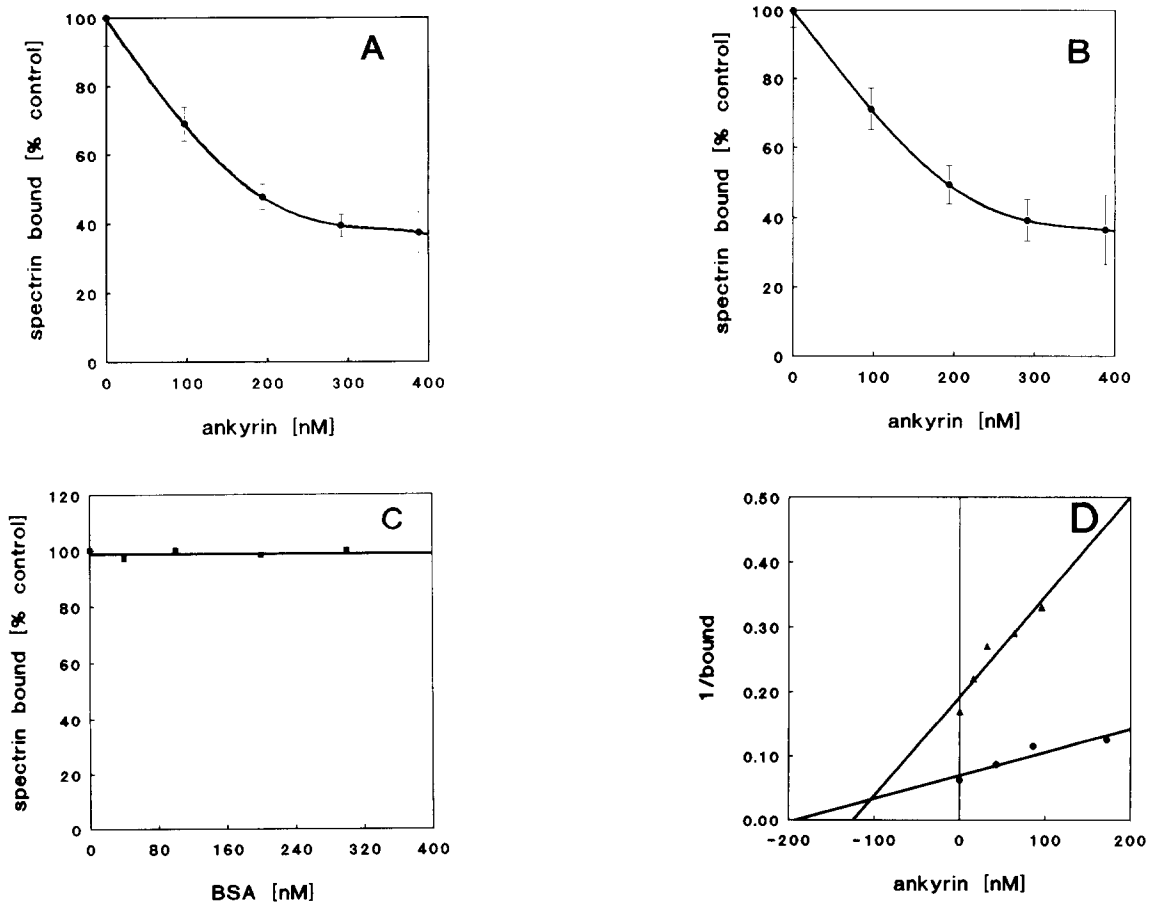

Fig. 4. Ankyrin inhibits binding of spectrin to liposomes prepared from phosphatidylethanolamine/phosphatidylcholine (60:40). Binding assay performed at pH 7.6 (A) and at pH 6.0 (B). Error bar = ±S.D. (C) Binding assay performed at pH 7.6 in the presence of indicated concentrations of bovine serum albumin. (D) Dixon [26] plot of the data obtained with the use of 125 and 225 nM spectrin in the sample. Other conditions as in A.

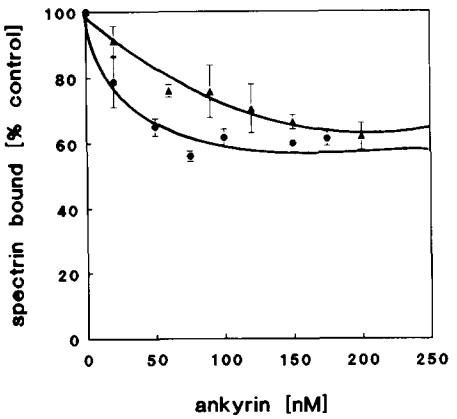

Fig. 5. Ankyrin partly dissociates spectrin–phosphatidylethanolamine-containing liposome complex. Binding assay performed at pH 7.6 (●———●) and at pH 6.0 (▲———▲). Experiments were performed as described in the text except that spectrin was pre-incubated 30 min with liposomes before ankyrin addition. Error bar = ±S.D.

at pH 7.6 is several times larger; the second reason could be the fact that after spectrin extraction, phospholipid asymmetry in the membrane and also the lipid microdomain organization could be disturbed [28,29]. Moreover, their control experiments were performed on 0.1 M acetic acid-treated membranes. It is possible that this type interaction was responsible for the curvilinear Scatchard plot obtained at pH 6.6 by these authors. Also the data from fluorescence experiments remain to be explained, since the lack of the red shift in the fluorescence maximum indicated many interacting sites [19]. It should be noted that the inhibition concerns 40–60% of phospholipid binding sites, therefore the remaining sites and the large size of ankyrin-binding site [30] might be responsible for the mentioned effect. Previously it was suggested [31] that proteolytic fragment of spectrin, which had strong affinity to hydrophobic agaroses, was a fragment of β subunit containing the ankyrin binding site. Kennedy et al. [30] showed that the ankyrin binding domain was formed by an entire 15 repeat segment of β subunit. This site also contains a highly conserved domain encompassing the first 33 residues of 15th repeat unit and extending through 3 residues of the 14th segment, which is highly conserved ($\sim 90\%$ identity) between red blood cell spectrin and nonerythrocyte spectrin (Ma, Y., Zimmer, W.E. and Goodman, S.R., personal communication). Physical properties of this fragment seem to be in good agreement with the phospholipid (phosphatidylethanolamine) binding ability. Of this sequence, 20 residues are hydrophobic according to the consensus hydrophobicity scale [32] ($\Delta\mu^\circ > 0$), seven are negatively and only two are positively charged. This could explain the difference in binding phosphatidylserine at pH 7.6 and 6.0. In addition, it should be noted that ankyrin as well as the spectrin binding site of ankyrin contains hydrophobic domains as was shown by Kahana et al. [33]. There are at least several situations in red blood cell when ankyrin is either absent, or its affinity for spectrin is reduced. Examples of the first situation are: in normal membrane some of spectrin tetramers are devoid of 9–12 mm globular particles [2,7], ankyrin is among the last components to continue to be synthesized and assembled to the membrane skeleton (for a review see [34]). Also, in genetic deficiency: erythrocytes of mutant mice, whose erythroblasts fail to synthesize ankyrin still accumulate $\sim 50\%$ of spectrin [35]. A well-known example of the second situation is the phosphorylation of ankyrin [36]. The physiological meaning of the described event would be that in certain situations phosphatidylethanolamine-rich membrane domains would serve as 'anchors' substituting ankyrin, ensuring the preservation of the mechanical properties of spectrin tetramer in the skeletal meshwork.

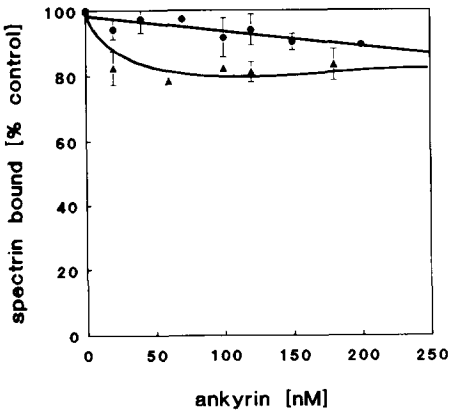

Fig. 6. The complex formation between spectrin and phosphatidylserine/phosphatidylcholine (60:40) liposomes is partly inhibited by ankyrin. Experimental details in the text. Binding assay at pH 7.6 (▲———▲) and at pH 6.0 (●———●). Error bar = ±S.D.

4. Acknowledgement

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5. References

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